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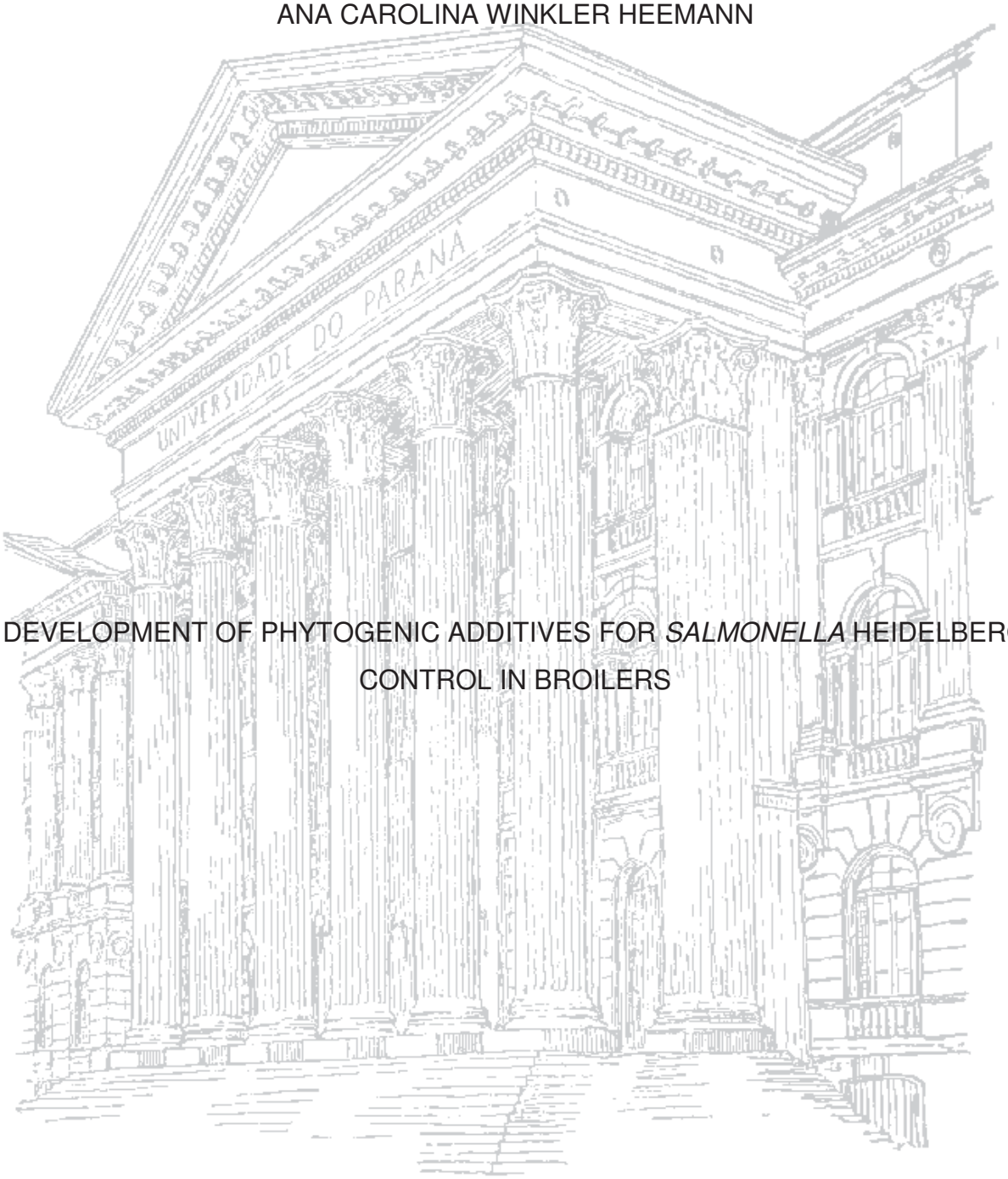

DEVELOPMENT OF PHYTOGENIC ADDITIVES FOR *SALMONELLA* HEIDELBERG CONTROL IN BROILERS

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ANA CAROLINA WINKLER HEEMANN

DEVELOPMENT OF PHYTOGENIC ADDITIVES FOR *SALMONELLA* HEIDELBERG CONTROL IN BROILERS

Tese apresentada ao curso de Pós-Graduação em Ciências Veterinárias, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências Veterinárias.

Orientadora: Profa. Dra. Elizabeth Santin
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RESUMO

A infecção de frangos de corte por *Salmonella enterica* serovar Heidelberg tem sido motivo de grande preocupação na avicultura mundial. Muitos pesquisadores reconhecem que os aditivos fitogênicos são considerados uma fonte importante de substâncias antimicrobianas e contribuir para o controle deste patógeno. Neste sentido, este trabalho foi desenvolvido buscando contribuir para o avanço das pesquisas com estes aditivos e inicia com a otimização do processo extrativo de polifenóis totais de erva-mate (*Ilex paraguariensis* A.St.Hil., Aquifoliaceae) utilizando enzimas em um delineamento experimental por resposta de superfície. Em seguida, utilizando técnicas radiológicas, avaliou-se a abertura do aditivo fitogênico microencapsulado em frangos de corte de 21 dias, buscando-se esclarecer se a micropartícula era resistente ao trato gastrintestinal, iniciando pela pressão mecânica exercida pelo papo e chegando ao final do trato, principalmente no ceco, local especialmente colonizado por patógenos devido aos processos fermentativos de carboidratos não digeridos. Finalmente, os aditivos fitogênicos microencapsulado (PAM) e livre (PAF) foram avaliados in vitro, com resultados promissores e também em experimento in vivo, no qual foi possível avaliar a eficácia antimicrobiana, histologia de fígado e ceco empregando a metodologia ISI e citometria de fluxo em sangue periférico. O aditivo fitogênico microencapsulado (PAM) não apresentou os resultados esperados, possivelmente por conter maltodextrina e menor teor de polifenóis totais em relação ao aditivo fitogênico livre (PAF). O aditivo fitogênico livre (PAF) apresentou potencial uso como antimicrobiano frente à cepa SH UFPR1, pois aos 21 dias, o PAF apresentou uma redução significativa da contagem de SH, apresentou valor de ISI significativamente menor no fígado e menor valor de CD8+ comparado ao controle positivo. Este resultado sugere um efeito antimicrobiano e antiinflamatório do aditivo fitogênico livre (PAF) em frangos de corte.

Palavras-chave: aditivo fitogênico, metodologia ISI, *Salmonella* Heidelberg, frangos de corte.

ABSTRACT

Infection of broiler chickens by *Salmonella* enterica serovar Heidelberg has been a major concern in poultry farming worldwide. Many researchers acknowledge that phytogenic additives are considered an important source of antimicrobial substances and contribute to the control of this pathogen. In this sense, this work was developed aiming to contribute to the advancement of the research with these additives and begins with the optimization of the extractive process of total polyphenols of yerba mate (*Ilex paraguariensis* St.Hil. Aquifoliaceae) using enzymes in an experimental design by response of surface. Then, using radiological techniques, phytogenic additive microencapsulated was then used in 21-day-old broiler chickens to clarify whether the microparticle was resistant to the gastrointestinal tract, starting with the mechanical pressure exerted by the gut and reaching the end of the treatment, especially in the cecum, a site specially colonized by pathogens due to fermentation processes of undigested carbohydrates. Finally, microencapsulated (PAM) and free (PAF) phytogenic additives were evaluated in vitro, with promising results and in vivo experiment, in which it was possible to evaluate the antimicrobial efficacy, liver histology and cecum using the ISI methodology and cytometry of flow in peripheral blood. Phytogenic additive microencapsulated (PAM) did not present the expected results, possibly because it contains maltodextrin and lower content of total polyphenols comparing to free phytogenic additive (PAF). The phytogenic additive free (PAF) presented a potential use as antimicrobial against SH UFPR1 strain, because at 21 days, PAF presented a significant reduction of the SH count, presented a significant lower ISI score in liver and lower result at blood CD8+ comparing to PC. This result suggests an antimicrobial and anti-inflammatory effect of the free phytogenic additive (PAF) in broilers.

Keywords: phytogenic additive, ISI methodology, *Salmonella* Heidelberg, broilers.

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GENERAL INTRODUCTION

Medicinal plants, essential oils and herbal extracts could be classify as phytogenic additives (PA) when used in animal feed. This additives have been used as antimicrobial (YANG et al., 2015) antioxidant (CHERIAN et al., 2013) and as a performance enhancer (PAPATSIROS et al., 2011). Essential oils and plant extracts are generally recognized as safe (GRAS) by the FDA (Food and Drug Administration) and some, especially those containing phenolic structures, present strong antimicrobial activity (SHEN et al., 2014). Many biological properties are attribute to these additives, including increased production of digestive secretions, stimulation of blood circulation, antioxidant properties, modulation of the intestinal microbiota, reduction of levels of pathogenic bacteria, possible improvement of immune status (BRENES; ROURA, 2010), improve zootechnical performance in livestock, and prevent and control enteric diseases in animals (PAPATSIROS et al., 2011).

Technologies for the modulation of the intestinal microbiota, such as the use of probiotics, prebiotics and phytogenic additives are promising for the development of new techniques against *Salmonella* in poultry farming (CHAMBERS et al., 2011). The administration of additives such as prebiotics (LOURENÇO et al., 2016), probiotics (KURITZA et al., 2013; MUNIZ et al., 2013; DONG et al., 2016), organic acids (GRILLI et al., 2007; PICKLER et al., 2012) MACHADO et al., 2014) and phytogenic additives (MUELLER et al., 2012; PICKLER et al., 2013) via feed and water has been extensively studied in poultry with the objective of improving intestinal health of broilers. Because of reducing intestinal pathogens, promoting a healthy intestinal environment, which would contribute to the reduction of carcass contamination at slaughter (PUVAČA et al., 2013), they are also described as the promising technologies to assure the microbiological quality in meat.

Despite of it, their use are still limited in regards to the mechanism of action and aspects of application. In addition, there are some concerns about that the botanical origin might change the composition of the plants and their extracts (ALLOUI et al., 2014).

Active principles of plant extracts are absorbed in the intestine by enterocytes and metabolized rapidly in the animal organism. The products of this metabolism are transformed into polar compounds by conjugation with the glucuronate and excreted in the urine. Other principles can still be eliminated by breathing. Rapid metabolism and the short half-life of the active compounds suggest that there is a minimal risk of accumulation in the tissues (KOHLERT et al., 2000).

To improve stability of these additives, microencapsulated phytogenic additives are being developed and evaluated, as an example a microencapsulated compound of essential oils of thyme, oregano, rosemary, rich in carvacrol, cinnamaldehyde, 1,8-cineol and pepper oleoresin. This additive showed a significant increase in egg production, lower mortality rates, a reduction in the number of dirty eggs and an increase in broiler breeders viability index, and it could be considered promising as a performance promoter (CHILANTE et al., 2012).

In addition to essential oils, vegetable extracts have been used to reduce pathogen contamination, like extracts of yerba mate (*Ilex paraguariensis*), that demonstrated a biocidal activity against *Salmonella* Enteritidis, but the *in vivo* evaluations showed that the supplementation with mate leaves was not effective in reducing the colonization of *Salmonella* Enteritidis in cecum. This difference could be explained by the bioavailability of the antimicrobial actives when in vitro extracts of yerba mate and the leaves of yerba mate *in vivo* (GONZALEZ-GIL et al., 2014).

Also green tea and its derivatives aided in reducing the microflora count in the cecum due to its "antibiotic-like" activity (KHAN, 2013). This action have been explained by high level of total polyphenols in yerba mate and green tea, according some authors may contribute to the maintenance of intestinal health by preserving the gut microbial balance through the stimulation of the growth of beneficial bacteria and the inhibition of pathogenic bacteria, exerting prebiotic-like effects (DUEÑAS et al., 2015).

Isolated substances, as trans-cinnamaldehyde and eugenol were performed *in vivo*, and trans-cinnamaldehyde being considered as a potential in the reduction of *Salmonella* Enteritidis (KOLLANOOR-JOHNY et al., 2012).

The aim of this thesis was to develop a phytogenic additive from green yerba mate (*Ilex paraguariensis* A.St-Hil., Aquifoliaceae) in the form microencapsulated (PAM) or free (PAF) to controlling *Salmonella enterica* serovar Heidelberg (SH) in broilers. In addition, it was studied they effect in immune and performance parameters of broilers.

In chapter 1 we present how the use of enzymes can improve polyphenols extraction of green yerba mate.

In chapter 2 we consider the process of microencapsulation as a form of protection of the phytogenic additives, as well as a controlled release form. In this study, we used radiological assessment to evaluate the site of release from this microparticle inside the gastrointestinal tract (GIT) of broilers.

In the final chapter, we present the results of *in vitro* and *in vivo* experiment evaluating phytogenic additives offered in different forms (PAM and PAF) on zootechnical performance, cecum and liver I see Inside histological evaluation and expression of immune cells in peripheral blood of broilers challenged or not with SH.

CHAPTER 1: ENZYME-ASSISTED EXTRACTION OF POLYPHENOLS FROM GREEN YERBA MATE

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ENZYME-ASSISTED EXTRACTION OF POLYPHENOLS FROM GREEN YERBA MATE

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SUMMARY

Enzyme-assisted extraction of bioatives from plants has been studied as an alternative green technology. Carbohydrases have been candidates to improve the extraction process of numerous bioactive compounds from plants. Polyphenols are secondary plant metabolites, generally involved in defense against different types of stress. Yerba mate (*Ilex paraguariensis* A. St.-Hil., Aquifoliaceae) is a natural source of this antioxidant compounds. The aim of this work was to evaluate enzyme-assisted extraction of polyphenols from green yerba mate to determine the best extraction conditions employing response surface methodology (RSM). The independent variables were temperature (33.2-66.8 °C), enzyme concentration (0-336 FGBU/100g), reaction time (19-221 minutes) and pH (2.82-6.18). The use of carbohydrases increased polyphenols extraction from about 38.67 % to 52.08 %. Our results showed that all independent variables were significant at linear level and temperature and pH were not significant on quadratic levels. Interaction of temperature and pH;

enzyme and reaction time; enzyme and pH were significant. The regression model showed determination coefficient (R^2) close 0.85 and adjusted close 0.45. Considering the results from this study and the industrial viability the best conditions to polyphenols extraction of green yerba mate are temperature of 50.00 °C, enzyme concentration of 168 FGB/100 g, reaction time of 120 minutes and pH 4.50. This study was the first RSM based optimized enzyme-assisted extraction of total phenolic compounds from green yerba mate.

Keywords: Carbohydrases, beta-glucanase, polyphenols, bioactive compounds, *Ilex paraguariensis*, response surface methodology.

1 INTRODUCTION

Ilex paraguariensis A. St.-Hil. is a plant that belongs to the family Aquifoliaceae and is widely distributed in Latin American countries, such as Brazil, Argentina, Paraguay and Uruguay (ANESINI et al., 2012). In these countries, the “mate” that is a tea made from the infusion of this plant’ dry leaves is considered one of the most consumed non-alcoholic beverages. The discovery of different substances with important biological activities from mate has also increased its consumption in other countries (MEJIA, DE et al., 2010). Due to this interest, there has been an increase in research related to the properties of yerba mate in the last 20 years (BRACESCO et al., 2011). In this studies, it has been shown that mate has antioxidant, antibacterial, antiviral, antitumor and antimutagenic properties (BASTOS et al., 2007; GEETHA et al., 2004; HECK et al., 2008). Regarding composition, the yerba mate extract presents flavonoids, vitamins such as vitamin A, B, C and E, among other

substances (MENINI et al., 2007). Furthermore, yerba mate's extract is very rich in polyphenols with levels even higher than levels in green tea and wines (GUGLIUCCI et al., 2009).

Nonetheless, extraction efficiency of polyphenols from yerba mate is influenced by herb particle size, solvents, pH, time, temperature and agitation (CRAFT et al., 2012). Conventional polyphenols extraction uses solvents such as ethyl alcohol or derived aqueous mixture and is used by many researchers (BAE et al., 2015). However, another method using enzymes has been developed and used to obtain plant-derived polyphenols, increasing the nutritional value of wines, juice preparations, extracting oils, among others substances (KAPASAKALIDIS et al., 2009). Enzymes are proteins that act in the degradation of compounds such as plant cell wall (plant cell wall degrading enzymes -PCWDE) such as cellulases, pectinases, xylanases and proteases (BEG et al., 2001) and can improve extraction of polyphenols from green yerba mate. For instance, cellulases act on the degradation of cellulosic compounds by catalyzing the breakdown of ß (1 → 4) glycosidic bonds that bind the glucose molecules needed for cellulose formation. All the aforementioned enzymes are produced by various microorganisms, including actinomycetes, bacteria and fungi (SHARMA et al., 2016). Enzyme-based extraction of bioactive compounds from plants is a potential alternative to conventional solvent-based extraction methods (PURI et al., 2012). For example, RSM based optimized enzyme-assisted extraction of antioxidant phenolics from underutilized watermelon (*Citrullus lanatus* Thunb.) (MUSHTAQ et al., 2015). Enzyme-assisted extraction has the advantage of being a green alternative to conventional methods (MUSHTAQ et al., 2017). The aim of this research was to evaluate the best

reaction time, temperature, enzyme concentration and pH to obtain polyphenols from green yerba mate using enzyme-assisted extraction for industrial use as an alternative to conventional solvent extraction. For this purpose, factorial experimental design and response surface methodology (RSM) were used since they have been a powerful statistical tool for studying the mutual interactions among variables over a range of values, while reducing the number of experimental trials (MOREIRA et al., 2014).

2 MATERIAL AND METHODS

2.1 Substrates

Leaves of yerba mate (*Ilex paraguariensis* A. St. Hil.) were collected in July 2016 from the region of São Mateus do Sul in the State of Paraná, Brazil.They were then processed in a traditional manner for enzymatic inactivation in a process called “sapeco”. For this, the yerba is submitted to temperature of 400 °C, at the inlet, and 65 °C, at the outlet, for the mean time of 8 seconds. Then it is dried in a dryer with temperature ranging from 90 and 110 °C, for about 3 hours, then it is ground to particle size of 2-3 mm. The specimen was identified and was deposited (voucher number 394262) at the Municipal Botanical Museum of Curitiba in the state of Paraná, Brazil. The commercial blend of enzymes Viscozyme® L Batch KTNO2241 (Novozymes, Denmark), was used in enzymatic hydrolysis with 100 FGBU/g, FGBU = Fungal Beta-Glucanase Units. This blend contains a range of carbohydrases consisting of arabanase, beta-glucanase, cellulase, hemicellulase and xylanase. Enzyme quantity (which hydrolyses beta-glucan to reducing sugars) corresponding to 1 µmol glicose per minute at pH 5.0 at 30 °C during 30 minutes. For the analysis

of polyphenols, Folin Ciocalteau reagent (Sigma-Aldrich, USA) and gallic acid standard (Chromadex, USA) were used.

2.2 Experimental design and statistical analysis

The influence of temperature, enzyme (FGBU/g), reaction time and pH on polyphenol extraction was evaluated on the basis of a CCRD 2^{4-1} + 8 axial points + 4 central points design (Table 1). This design was used to obtain properties such as randomness, rotatability and orthogonality for best fitting (MYERS and MONTGOMERY, 2002). The quality of the polynomial model equation was determined statistically by the coefficient of determination R^2. *P*-values lower than 0.05 were considered to be statistically significant. For the ANOVA analysis, pure error was used. All statistical analyses were performed with Statistica 13.2 software (Statistica, 2016).

Table 1: Variable levels of the CCDR 2^{4-1} + 8 axial points + 4 central points design to evaluate polyphenol extraction from green yerba mate

Variable	Level				
	-1.68179	-1	0	+1	+1.68179
(1) Temperature (°C)	33.2	40.0	50.0	60.0	66.8
(2) Enzyme (FGBU/100g)	0	68	168	268	336
(3) Reaction time (min)	19	60	120	180	221
(4) pH (25°C)	2.82	3.50	4.50	5.50	6.18

2.3 Assays

In the surface response optimization assays, 20 Erlenmeyer flasks 250 mL were used to mix 4 g of yerba mate + 100 mL of water and enzyme, as described in table 1. The biotechnological experiment was conducted in

Dubnoff-type Metabolic Bath (Novatécnica, Brazil). The pH was evaluated by direct reading in pH meter Q400A (Quimis, Brazil) according to procedure described in the Brazilian Pharmacopoeia, 2010.

2.4 Total polyphenol assay

The evaluation of the total polyphenol concentration in dried leaves of green yerba mate (*Ilex paraguariensis* A. St. Hil.) and enzymatic extracts was determined following the European Pharmacopoeia, 8th edition (European Pharmacopoeia, 2014). We used Folin Ciocalteau (FC) reagent and a spectrophotometer reading SP-1105 (Spectrum, China) at 760 nm, using gallic acid as standard. This assay is based on the chemical reduction of the reagent containing sodium molybdate and sodium tungstate, which reacts with phenols and other substances such as ascorbic acid, aromatic amines, sugars and xanthines (SINGLETON et al., 1998).

3 RESULTS AND DISCUSSION

The experiments were performed on a c*entral composite* rotatable design rotational (CCRD) considering the follow model: CCRD 2^{4-1} + 8 axial points + 4 central points design. The coded values of the experimental factors and their levels for DCCR are presented in Table 1. The incomplete design was carried out in a random manner in order to minimize the effect of non-controlled variables. A 4-factor with 5-level design is suitable for exploring quadratic response surface and constructing second-order polynomial equation. Analysis of results conducted according CCRD made it possible to obtain the following equation:

TP = 7.81077-0.110909*Temp.-0.000107837*Temp.*Temp.-0.270076*Enzyme-0.163313*Enzyme*Enzyme+0.012548*Time-3.54366e-005*Time*Time-1.73507*pH-0.00316604*pH*pH+0.0076881*Temp.*Enzyme-6.875e-005*Temp.*Time+0.0323881*Temp.*pH+0.00153333*Enzyme*Time+0.108952* Enzyme* pH+0.000425*Time*pH

Total polyphenol extraction results on different temperatures (°C), enzyme concentration (FGBU/100g), reaction time (min) and pH are presented in Table 2. According to Pareto chart (Figure 1) and ANOVA analysis (Table 3), the effects of all variables were significant ($p \leq 0.05$) on linear levels while temperature and pH were not significant on quadratic levels. The interaction between (1) temperature and (4) pH; (2) enzyme concentration and (3) reaction time; (2) enzyme concentration and (4) pH were also significant ($p \leq 0.05$). The regression model presented a determination coefficient (R^2) close to 0.85 and adjusted coefficient close to 0.45. If the adjusted R-squared decreases when a predictor improves the model less than expected by chance, the lower value obtained in this study can be justified due to the lack of difference in quadratic values for the variables temperature and pH. This commercial enzyme, Viscozyme® L, has been previously used to optimize polyphenol extraction in green tea (*Camellia sinensis*) and was the best choice among other commercial available enzyme like Celluclast®, Cytolase®, Econase®, Pectinex®, Rapidase® and Ultraflo® (HONG et al., 2013). The same enzyme has been also used for a pre-treatment of green tea residues and to improve the extraction of metabolites and applied at mild conditions (temperature below 60 °C), since it preserves the integrity of other side products such as pigments and polyphenols (ZHANG et al., 2016). Many studies have demonstrated that the use of enzymes increases

the amount of phenolic compounds as well as antioxidant activity (CERDA et al., 2013). The enzymatic extraction of polyphenols occurs through the hydrolytic degradation of polysaccharides from the plant cell wall, which retains the phenolic compounds in a network of polysaccharides and lignin bounded by hydrogens or hydrophobic bonds. Another mechanism of enzymatic action is direct catalysis of the disruption of ether and / or ester bonds between the phenols and polymers of the plant cell wall (PINELO; MEYER, 2008).

Table 2: Total Polyphenols (mg GAE/g) from green yerba mate under different conditions of temperature (°C), enzyme concentration (FGBU/100g), reaction time (min) and pH.

Run	Temp. (°C)	Enzyme concentration (FGBU/100g)	Reaction time (min)	pH	Total Polyphenols (mg GAE/g)
1	60.00	268	180.00	3.50	3.180 ± 0.005
2	60.00	268	60.00	3.50	2.745 ± 0.022
3	60.00	68	180.00	5.50	3.241 ± 0.066
4	40.00	268	60.00	5.50	2.281 ± 0.018
5	60.00	68	60.00	5.50	3.072 ± 0.018
6	40.00	68	180.00	3.50	2.890 ± 0.005
7	40.00	268	180.00	5.50	2.983 ± 0.080
8	40.00	68	60.00	3.50	2.658 ± 0.033
9	33.20	168	120.00	4.50	2.435 ± 0.055
10	66.80	168	120.00	4.50	3.400 ± 0.066
11	50.00	0	120.00	4.50	2.084 ± 0.070
12	50.00	336	120.00	4.50	2.890 ± 0.036
13	50.00	168	19.00	4.50	1.803 ± 0.022
14	50.00	168	221.00	4.50	3.371 ± 0.044
15	50.00	168	120.00	2.82	2.788 ± 0.005
16	50.00	168	120.00	6.18	3.090 ± 0.013
17 (CP)	50.00	168	120.00	4.50	3.168 ± 0.078
18 (CP)	50.00	168	120.00	4.50	3.125 ± 0.013

19 (CP)	50.00	168	120.00	4.50	3.255 ± 0.093
20 (CP)	50.00	168	120.00	4.50	3.130 ± 0.063

mg GAE/g = mg gallic acid equivalent/g.

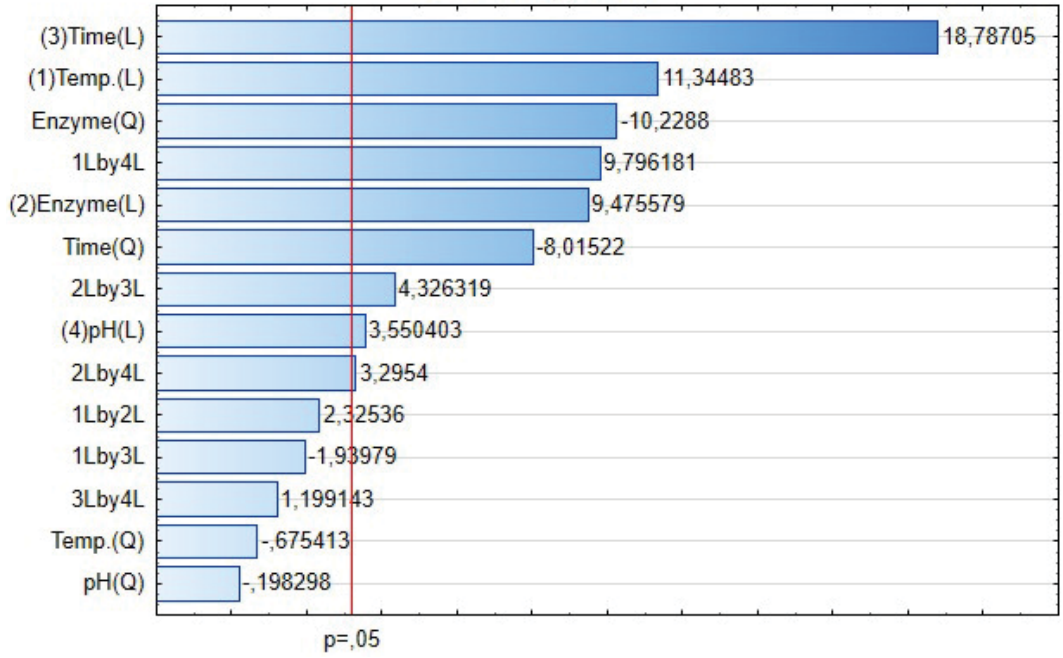


Figure 1: Pareto chart for the effects calculated from the responses of CCRD 2^{4-1} + 8 axial points + 4 central points design to evaluate polyphenol extraction from green yerba mate considering (1) temperature (°C), (2) enzyme concentration (FGBU/100g), (3) reaction time (min) and (4) pH. ($p<0.05$).

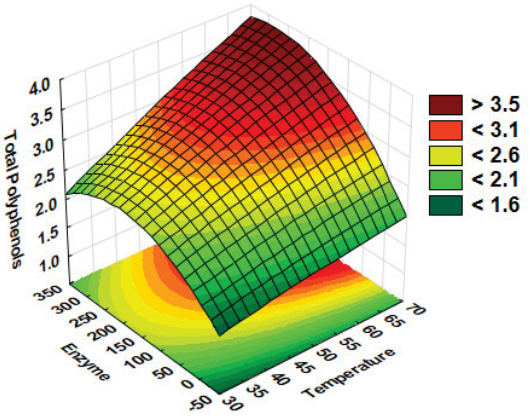
TABLE 3: Analysis of variance (ANOVA) for CCRD 2^{4-1} + 8 axial points + 4 central points design to evaluate polyphenol extraction from green yerba mate

Factor	SS	Df	MS	F	P
(1) Temperature (L)	0.465612	1	0.465612	128.7052	0.001469
(1) Temperature (Q)	0.001650	1	0.001650	0.4562	0.547813
(2) Enzyme (L)	0.324818	1	0.324818	89.7866	0.002492
(2) Enzyme (Q)	0.378511	1	0.378511	104.6285	0.001992
(3) Reaction time (L)	1.276867	1	1.276867	352.9532	0.000329
(3) Reaction time (Q)	0.232413	1	0.232413	64.2438	0.004054
(4) pH (L)	0.045602	1	0.045602	12.6054	0.038080
(4) pH (Q)	0.000142	1	0.000142	0.0393	0.855489

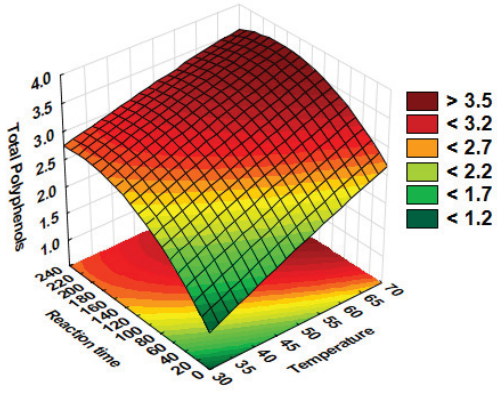
1L by 2L	0.019562	1	0.019562	5.4073	0.102581
1L by 3L	0.013612	1	0.013612	3.7628	0.147742
1L by 4L	0.347170	1	0.347170	95.9652	0.002261
2L by 3L	0.067712	1	0.067712	18.7170	0.022767
2L by 4L	0.039287	1	0.039287	10.8597	0.045892
3L by 4L	0.005202	1	0.005202	1.4379	0.316550
Lack of Fit	0.508290	2	0.254145	70.2511	0.003023
Pure Error	0.010853	3	0.003618		
Total SS	3.596007	19			

Variables and interactions in bold express significance ($p<0.05$).
$R^2 = 0.85563$ Adj. = 0.45141

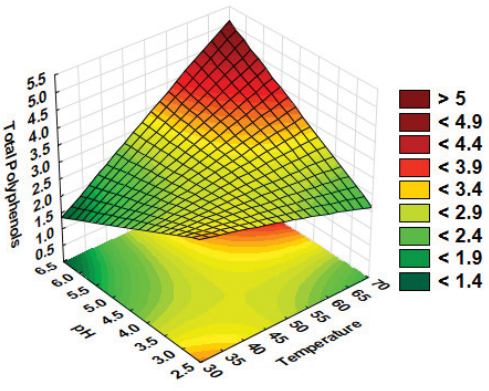
(a)



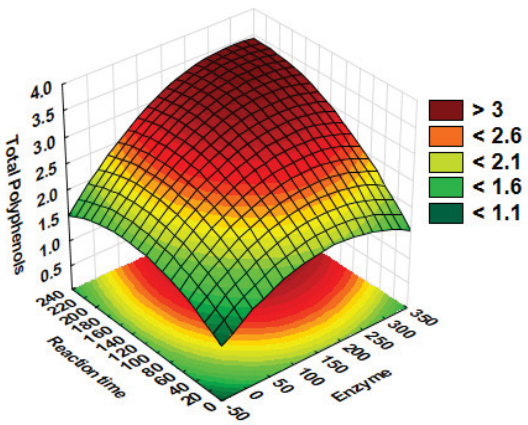
(b)



(c)



(d)



(e) (f)

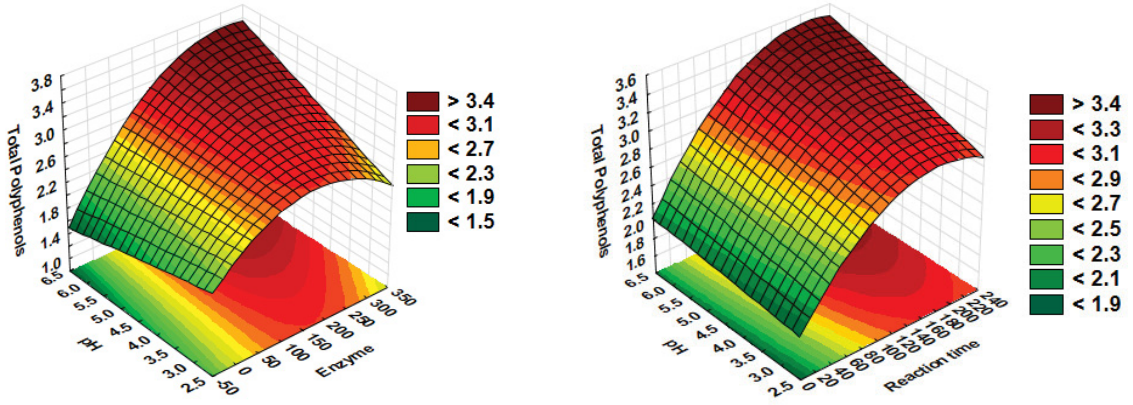


Figure 2: Response surfaces for CCRD 2^{4-1} + 8 axial points + 4 central points design to evaluate polyphenol extraction from green yerba mate. (a) temperature (°C): enzyme concentration (FGBU/100g). Fixed reaction time 120 minutes and fixed pH 4.5; (b) temperature (°C): reaction time (minutes). Fixed enzyme concentration 168 FGBU/100g and pH 4.5; (c) temperature (°C): pH. Fixed enzyme concentration 168 FGBU/100g and reaction time 120 minutes; (d) reaction time (min): enzyme concentration (FGBU/100g). Fixed temperature 50 °C and pH 4.5; (e) pH: enzyme concentration (FGBU/100g). Fixed temperature 50 °C and reaction time 120 minutes; (f) pH: reaction time (min). Fixed temperature 50 °C and enzyme 168 FGBU/100g.

The presence of an enzyme increased polyphenol extraction of dry green leaves of yerba mate (Figure 2a). Regarding enzyme concentration, it was possible to observe that the concentration of 336 (FGBU/100 g) (Table 2, run 12) increased polyphenol quantity in 38.67 % in relation to extraction without enzyme, under the same experimental conditions (Table 2, run 11). Polyphenols increase was observed in central points in which intermediate enzyme concentration of 168 FGBU/100 g of dry green leaves of yerba mate was used, in the same experimental condition of run 11 and 12; in this scenario, polyphenols extraction increased 52.08% in average (four central points) (Table 2, run 17-20) in relation to run 11 (without enzyme). This effect could be explained by enzyme/substrate relation, in which the excess of enzyme was used in quadratic levels and because the quantity of total polyphenols

decreased in this condition. Therefore, the best enzyme concentration was 168 FGBU/100 g of dry green leaves of yerba mate for industrial viability. Phenolics and flavonoids extraction conditions of roasted yerba mate leaves (*Ilex paraguariensis* A. St.-Hil., Aquifoliaceae) was obtained using Response Surface Methodology (BASSANI et al., 2014). Total polyphenol extraction of yerba mate using ethanol has been previously studied and the best parameters were: reaction time of 103 min, extraction temperature of 71 °C and ethanol concentration of 61% (BAE et al., 2015). Enzyme-assisted extraction of secondary metabolites is considered a green and clean technology unlike to conventional solvents like alcohols (methanol, ethanol), acetone, diethyl ether and ethyl acetate that are used in polyphenol extraction despite several disadvantages: besides a possible hazardous effect on human health due to remaining solvent residues in the final products, the disposal of these solvents can also cause environmental problems (MOJZER et al., 2016). Enzymatic extraction is an alternative for secondary metabolic extraction, for example, enzymatic extraction pilocarpine from *Pilocarpus jaborandi* was 3.08 fold greater than in control treatment (CHO et al., 2013). In green tea, enzymatic extraction showed significant increase of 4–15 % in the total polyphenol content of green tea extract when compared to non- enzymatic treatment (HONG et al., 2013). In Figure 2b, it is possible to observe that polyphenol extraction was improved with higher temperature: in temperature of 66.80 °C total polyphenol quantity increased 7.27 % in relation to central point that uses a temperature of 50 °C. Therefore, a difference of 16.8 °C is justified since it improves polyphenol extraction. According to the literature, the enzymatic activity can be decreased by protein denaturation, that is, an irreversible process of loss of primary

structure with associated covalent bond cleavage in temperatures above 80 °C (DANIEL et al., 1996). Nonetheless, the higher temperature did not show a significant effect with pH bellow 3.5 (Figure 2c). This result is in agreement with current optimal conditions in which ideal pH is 3.3-5.5 (HONG et al., 2013). Reaction time was a significant effect on polyphenols extraction of green yerba mate (Figure 2d), although polyphenol quantity increased only 6.35 %. Enzyme action is pH dependent (BISSWANGER, 2014) and the same was observed in this study since there was a significant interaction of (2) enzyme concentration and (4) pH (Figure 1 and Table 3). The change in pH alone was not significant to increase polyphenol extraction of yerba mate (Figure 2e), but an interaction between enzyme concentration and pH was observed to produce such effect. Neither the pH over short reaction time extraction nor the interaction between pH and reaction time produced a significant difference (Figure 2f). The optimization algorithm allowed the elaboration of profiles for predicted response values and desirability functions (Figure 3); the red line indicates best conditions and desirability score of 1.0 indication: temperature of 50.00 °C, enzyme concentration of 168 FGB/100 g, reaction time of 120 minutes and pH

4.50.

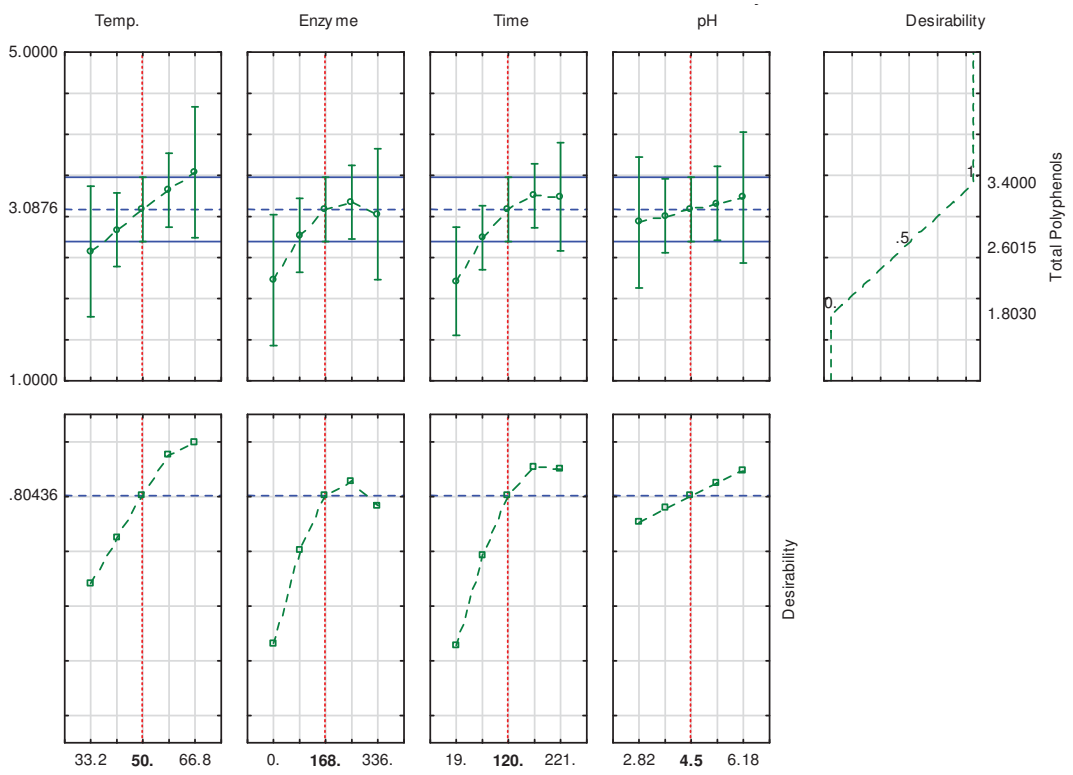


Figure 3: Profiles for predicted values and desirability function for the maximum extraction efficiency of total polyphenols from green yerba mate. Dotted red lines indicate the optimization values.

4. CONCLUSION

In this work CCRD 2^{4-1} + 8 axial points + 4 central points design was used to evaluate extraction variables (temperature, enzyme concentration, reaction time and pH) and possible interactions on total polyphenol extraction from dried leaves of green yerba mate. By using response surfaces, contour curves and derivation techniques it was possible to obtain a definition of the optimum application conditions for the investigated enzyme. It was concluded that the effects of all studied variables (temperature, enzyme concentration, reaction time and pH) were significant on linear levels although temperature and

pH were not significant on quadratic levels, parameters that can be explained by optimal enzymatic conditions. The interaction between temperature and pH, enzyme concentration and reaction time and enzyme concentration and pH were significant. Based on the results of this study an taking into account industrial viability we suggest that the optimal conditions for total polyphenols extraction from green yerba mate are temperature of 50.00 °C, enzyme concentration of 168 FGB/100 g, reaction time of 120 minutes and pH 4.50. Enzymatic-assisted extraction of polyphenols from green yerba mate was considered a good alternative to conventional solvent extraction since enzymes are a renewable that further contributes to sustainable practices.

CONFLICT OF INTEREST

There is no conflict of interest.

ACKNOWLEDGEMENTS

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CHAPTER 2: RADIOLOGICAL ASSESSMENT OF MICROENCAPSULATED PHYTOGENIC ADDITIVE RELEASE SITE IN BROILERS

Radiological assessment of microencapsulated phytogenic additive release site in broilers

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ABSTRACT

Noninvasive techniques such as x-ray have been used for the evaluation of the release of substances and evaluation of intestinal transit. Knowledge of the site of release of additives such as organic acids, phytogenic additives, prebiotics, and probiotics is necessary for such additives to have their optimized efficacy. The performance of additives from the cecum, for example, may contribute to the reduction of pathogens, such as *Salmonella* sp. The aim of this work is to use radiological evaluation of the intestinal transit time of microencapsulated phytogenic additive containing barium sulfate in broilers. The experiment was divided into two stages. In the preliminary stage, three birds 21 days old received 3 different diets, D_1 - feed; D_2 - feed + barium sulfate (10%) and D_3 - feed + microparticles (10%). The birds that received D_1 did not present any contrast at the evaluated times since they only received the feed. The 21 days old birds that received D_2 and D_3 presented contrasted images in the duodenum region for the first hour. The most significant difference was observed at the time 2 hours and 30 minutes when only the birds that received D_2 presented barium sulfate contrast in the crop. The bird that received D_3 did not present contrast for the same time, indicating that the microencapsulation process was

efficient in the conduction of the phytogenic additive and the barium sulfate to the intestinal portion, without complete opening in the region of the crop. In the second stage, twenty-four birds with 21 days of age were divided into three groups of eight birds and received the same diets of the preliminary stage. The results showed that the diet significantly influenced the measurement of the crop and the differences, in contrast, were the same as the preliminary stage. Radiology analysis with the use of radio-opaque material allows evaluation of intestinal transit time and the visualization of microparticle contents release site in broilers.

Key words: intestinal health, microencapsulation systems, controlled release, gastrointestinal tract, noninvasive techniques.

1 INTRODUCTION

The administration of additives such as prebiotics (LOURENÇO et al., 2016), probiotics (DONG et al., 2016), organic acids (GRILLI et al., 2007) and phytogenic additives (MUELLER et al., 2012) via feed and water has been extensively studied in poultry with the objective of improving intestinal health of broilers.

Phytogenic additives are plant derived products used in animal feed for antimicrobial effect (YANG et al., 2015) antioxidant (CHERIAN et al., 2013) and as a performance enhancer (PAPATSIROS et al., 2011). Essential oils and plant extracts are generally recognized as safe (GRAS) by the FDA (Food and Drug Administration) and some, especially those containing phenolic structures, present strong antimicrobial activity (SHEN et al., 2014). Technologies for the

modulation of the intestinal microbiota, such as the use of probiotics, prebiotics and phytogenic additives are promising for the development of new techniques against *Salmonella* in poultry farming (CHAMBERS et al., 2011). During the last 15 years, phytogenic additives have attracted attention in animal nutrition, mainly as an alternative to growth promoting antibiotics; the effectiveness of these additives in the diet of broilers depends on factors such as composition and levels of these additives in the diet, genetics of the bird and the remaining composition of the feed (PUVAČA et al., 2013).

In 2006, the use of antibiotics to improve performance has been banned in the European Union; since then, it has only been allowed as therapeutics and the search for natural alternatives with similar effectiveness has been intensified (AMAD et al., 2011). An attempt to improve the stability of these additives and to promote the appropriate delivery site was employing the technique of microencapsulation of substances (GBASSI; VANDAMME, 2012).

Microencapsulation is a technology for retaining substances of interest within another material that protects, controls and releases the active substance. This protective structure can be of the beads type (active distributed in the matrix) or capsules (active envolved by the matrix (DIAS et al., 2015). This technology has been use to protect the additives from the first pass metabolism and to modulate the site of opening and absorption at enteral release (MOHAMMADI GHEISAR et al., 2015). In birds, the evaluation of intestinal transit time is documented using dyes such as iron oxide and chromium oxide (KIM et al., 2013; SILVA et al., 2013; ANDREOTTI et al., 2004). However, it is difficult to establish techniques that evaluate the site of release of the content of the microparticles.

For this evaluation, the radiological study with the inclusion of contrast barium sulfate was chosen as methodology in this study. Radiological studies in birds are infrequent. Among these few, we can highlight the X-ray (RX) and contrast with barium sulfate of five *Ramphastos toco* birds known as toucan-toco; the birds were radiographed in the ventrodorsal position and the final marker release time was 334 ± 20 minutes (SILVA et al., 2012). Also using barium sulfate as contrast, the entire duodenum of six birds of the *Amazona aestiva* species was filled at 60 minutes (VINK-NOOTEBOOM et al., 2003). Radiology was also used for the morpho-anatomical evaluation of the species *Apteryx australis mantelli* (BEALE, 1985). The intestinal transit time of domestic pigeons (*Columba livia*) has been evaluated with the use of polyethylene spheres employed with barium sulfate and barium sulfate in suspension; the use of this technique was not recommended since the transit time was higher with spheres of 1.5 mm of diameter that were retained in the ventricle (BLOCH et al., 2010). Radiological evaluation with barium sulfate showed an intestinal transit time of 555 ± 126.4 minutes in *Caracara plancus* (SANTOS et al., 2011). Radiological studies can provide information on the transit in the gastrointestinal tract (GIT) and are useful for detailing its anatomical structure (WILDING et al., 2001).

The aim of the present work is to use radiological evaluation of the release site of microencapsulated phytogenic additive containing barium sulfate, as well as to monitor the intestinal transit time using contrasted microparticles by means of RX images in broilers as a non-invasive technique.

2 MATERIALS AND METHODS

This experiment was approved by the Institutional Animal Use Ethics Committee of the Agricultural Sciences Campus of the Federal University of Parana (Protocol 038/2016).

2.1 Birds, Feed and Microparticles

In preliminary stage, three birds with 21 days were used for a qualitative study. They were, from the Experimental Farm of the Federal University of Paraná, located in the municipality of Pinhais, in the state of Paraná, Brazil and were fed with ration formulated according to the age of the birds. In the second stage, twenty-four birds with 21 days were used for the quantitative study. The microparticles (MP) of phytogenic additive containing barium sulfate (Synth, Brazil) were prepared by spray drying process and assigned to the experiment (Heide, Brazil).

2.2 In vitro experiment

In order to evaluate the radiological images, an in vitro experiment was carried out evaluating the feed and the additives: (A) Feed; (B) Feed + barium sulfate powder (10%); (C) Feed + microparticles containing barium sulfate (MP) (10%).

The concentration of 10% barium sulfate in the diet was established for best contrast visualization. The same concentration of microparticles was used to maintain the standard and not to change palatability. The use of powdered barium sulfate incorporated in the feed facilitates administration to birds

compared to the use of the commercial suspension of the product that requires administration in liquid form, compromising handling.

2.3 In vivo experiment, preliminary stage

Three birds were fasted for 2 h and then received feed containing different additives: D_1 - feed; D_2 - feed + barium sulfate (10%) and D_3 - feed + MP (10%). The birds were radiographed at the ventrodorsal position and lateral decubitus position after 1 hour and every 30 minutes until the final time of 150 minutes, when barium sulfate was detected in the excreta. The birds were contained in the appropriate position to obtain the images, and the volunteers used the individual protection equipment recommended for this analysis. The images were obtained by a veterinarian radiologist who has accompanied the whole experiment.

2.4 In vivo experiment, second stage

Twenty-four birds were randomly divided into 3 groups with 8 replicates. After 2 hours of fasting they received feed containing different additives for 15 minutes: D_1 - feed; D_2 - feed + barium sulfate (10%) and D_3 - feed + MC (10%). After feeding was cut off, the birds were radiographed only at the lateral decubitus position every 30 minutes until 150 minutes of experiment, when barium sulfate was detected in the excreta.

2.5 Radiological images

A computerized radiography (CR) technique was used with the Medicor Budapest X-ray device of the brand Neo-Diagnomax with 500 mA power with subsequent scanning - AGFA, model CR-30 located at the Veterinary Hospital

of the University Federal do Paraná, at Curitiba, state of Paraná, Brazil. The examinations were carefully evaluated using a high-resolution monitor to verify the correct positioning of the birds. Settings were 48 kV, 8mA and one second. The images were salved in DICOM format and analyzed using RadiAnt DICOM Viewer version 4.2.1.17555. The same technic was used for all birds and times.

2.6 Optical microscopy images

Microparticle images were obtained using an optical microscope model DM 1000 LED (Leica, Germany) with Leica Application Suite version 4.8.0 software, located in the Laboratory of Microbiology and Ornithology at the Federal University of Paraná (LABMOR-UFPR), Curitiba, Paraná state, Brazil.

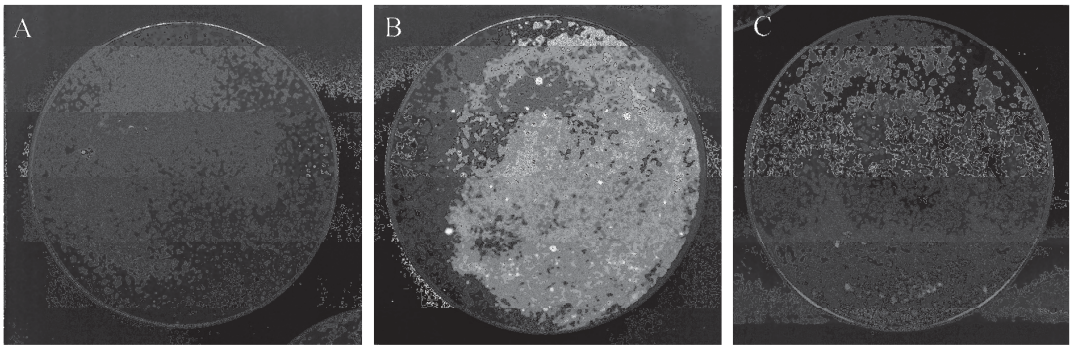
2.7 Statistical analysis

The parametric data were compared using analysis of variance (ANOVA) and Tukey's test. Data were analyzed using the statistical software Statistix version 9.0.

3 RESULTS AND DISCUSSION

In figure 1, we can observe that: (A) Feed: no contrast, as expected; (B) Feed + barium sulfate powder (10%): contrast, since the preparation was performed only by mixing the feed with barium sulfate in 10% concentration; (C) Feed + microparticles containing barium sulfate (MP) (10%): no contrast,

proving that the wall material and the microencapsulation process were efficient in hiding the radiopaque material.



(A) Feed (B) Feed + Barium Sulfate (10%) (C) Feed + MP (10%)

Figure 1. X-ray images in vitro for verification of radiopaque material.

The microparticles of phytogenic additive containing barium sulfate (MP) were predominantly spherical and polydisperse forms under optical microscopy, characteristics of products obtained by spray drying (Figure 2). The same was observed in another study in which microparticles of green tea were produced and evaluated by electron microscopy, also presenting spherical shape (WANG et al., 2016). Spherical microparticles were also obtained in the drying of blackberry (*Rubus* sp.) with maltodextrin using spray drying (FERRARI et al., 2012).

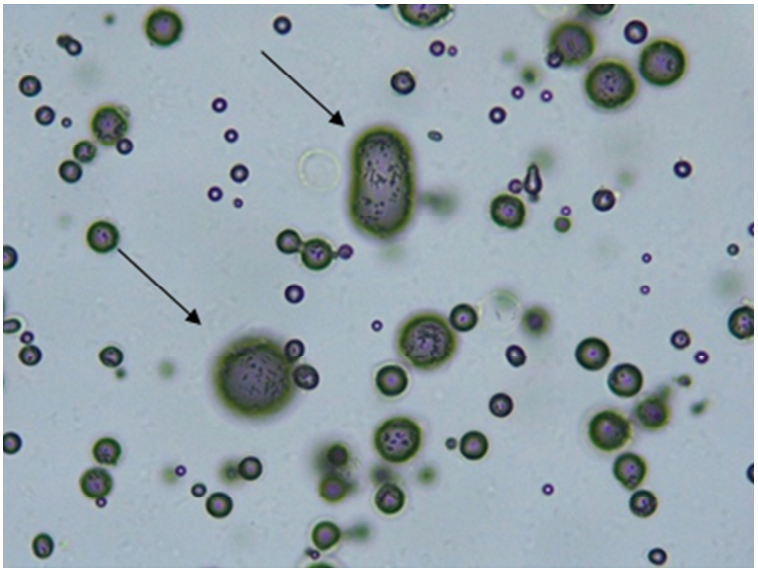


Figure 2. Microparticles of phytogenic additive microencapsulated containing barium sulfate by optical microscopy in an increase of 40X.

In figure 3 shows the ventrodorsal radiological images obtained in the three birds, according to the received feed, being D_1 - feed; D_2 - feed + barium sulfate (10%) and D_3 - feed + MP (10%). The D_1 bird did not present contrast at any of the evaluated times since it received control ration. The birds that received D_2 and D_3 presented images contrasted in the duodenum region in the first hour. The greatest difference was observed in the time 150 minutes, when only the bird that received D_2 presented contrast of barium sulfate in the chat. The bird that received D_3 did not present the contrast at the same time, indicating that the wall material used in the microencapsulation process was efficient in the conduction of the phytogenic additive and the barium sulfate to the intestinal portion, without complete release in the region of the crop. If the microparticles had completely ruptured in the crop, it would be impregnated with barium sulfate, as it was observed for the bird that received D_2. The experiment was concluded at 150 minutes since the birds receiving D_2 and D_3 presented barium sulfate in the excreta.

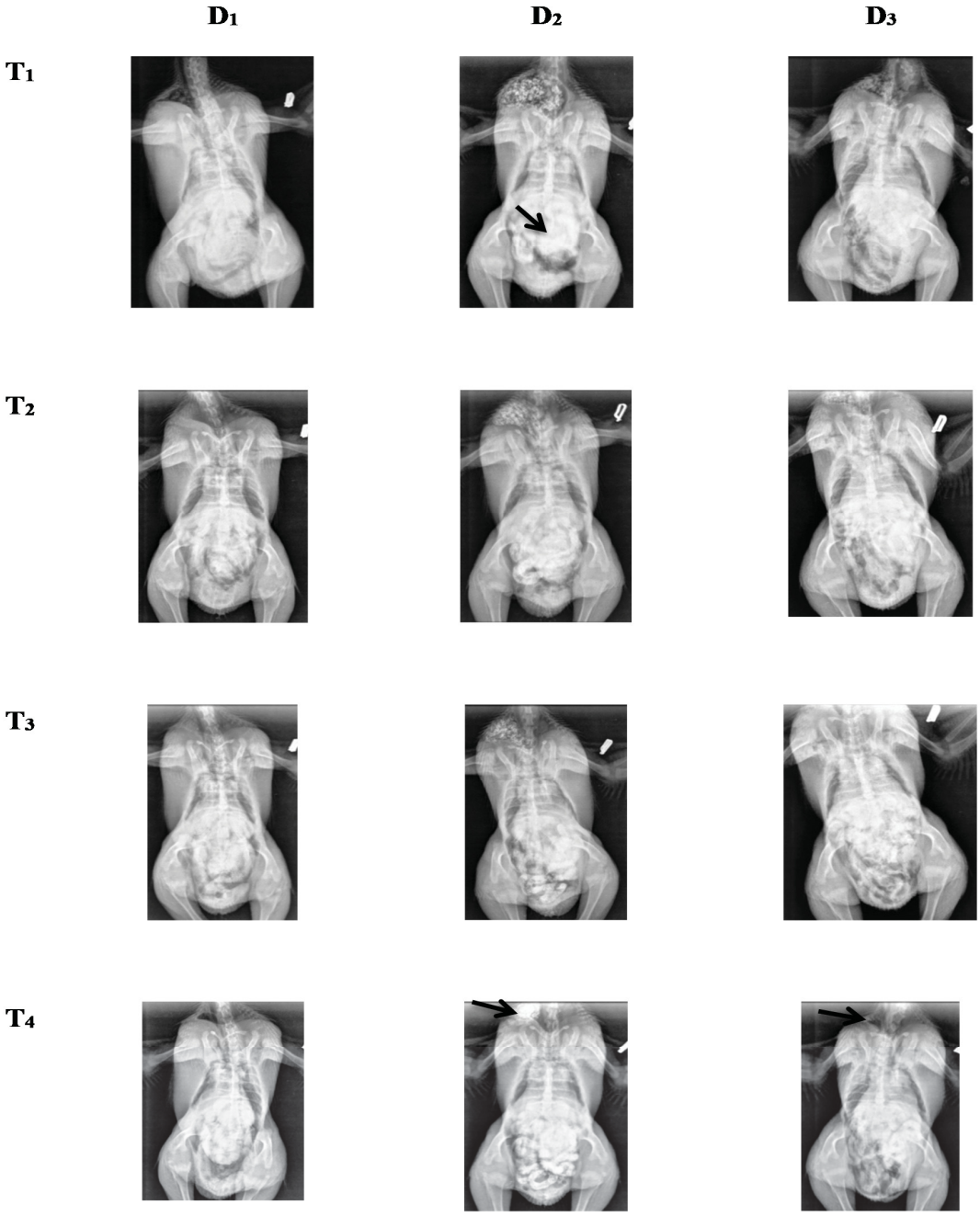


Figure 3. Radiological assessment broilers 21 days of age, ventrodorsal.
D_1 – feed (no contrast present); D_2 - feed + barium sulfate (10%) (contrast in GIT on 60m and contrast in crop on 150m); D_3 - feed + microparticle with barium sulfate (10%) (no contrast in crop and contrast in GIT in all times evaluated). Times assessed: T_1 - 60 m; T_2; 90 m; T_3 - 120 m and T_4 - 150 m.

The use of barium sulfate as a contrasting agent in radiology and as a marker for evaluation of intestinal transit time in the veterinary area has been well documented (SHAIKH et al., 2015), and new studies have been carried out in an attempt to improve the technique using barium sulfate nanoparticles encapsulated in dextrin as contrast agent or controlled release marker (MEAGHER et al., 2013).

In table 1 shows the crop length (cm) according to diet and evaluation time. The measurements from animals from the diets D_1 - feed and D_2 - feed + barium sulfate (10%) were very similar. The measurements of the diet D_3 - feed + microparticles with barium sulfate (10%), however, were smaller at 30 min, 60 min, 90 min and 120 min, being equal to the others only at the final evaluation time (150 min). This demonstrates that, for some reason still to be clarified, the evaluated birds did not ingest the same volume of the D_3 diet, possibly due to difference in odor and/or taste of the feed containing the phytogenic additive.

Table 1: Crop length (cm) of images obtained in *in vivo* experiment, second stage

Time	D_1	D_2	D_3
30 min	2,9875 ± 0,3032 aA	3,4838 ± 0,3137 aA	1,3500 ± 0,0908 bA
60 min	2,5275 ± 0,3271 bAB	3,4813 ± 0,3076 aA	1,1613 ± 0,0925 cA
90 min	2,0525 ± 0,3047 abAB	2,8113 ± 0,3235 aA	1,2313 ± 0,0830 bA
120 min	1,8225 ± 0,2628 abAB	2,3550 ± 0,2994 aAB	1,0713 ± 0,0794 bA
150 min	1,5850 ± 0,3291 aB	1,5500 ± 0,1911 aB	1,0538 ± 0,0888 aA

Tukey's test P value <0.05. Lowercase letter refers to the line and the uppercase letter refers to the column.

4 CONCLUSION

Radiology analysis using radio-opaque material, such as barium sulfate powder in microencapsulation systems, allowed the evaluation of intestinal

transit time in birds and to follow the content release site of the phytogenic additive microparticles.

The technique may be recommended in controlled release studies of phytogenic additives, organic acids, probiotics, antibiotics and others, thus contributing to improve quality of tests regarding avian health and, as a consequence, reduction of the risk of transmitted diseases to consumers of poultry products.

CHAPTER 3: EFFECT OF GREEN YERBA MATE PHYTOGENIC ADDITIVES ON *SALMONELLA ENTERICA* SEROVAR HEIDELBERG (SH) UFPR1 STRAIN CONTROL, CECUM AND LIVER I SEE INSIDE HISTOLOGICAL EVALUATION AND EXPRESSION OF IMMUNE CELLS IN PERIPHERAL BLOOD OF BROILERS

Effect of green yerba mate phytogenic additives on *Salmonella enterica* serovar Heidelberg (SH) UFPR1 strain control, cecum and liver I see Inside histological evaluation and expression of immune cells in peripheral blood of broilers

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SUMMARY

The phytogenic additives have been used to control *Salmonella enterica* serovar Heidelberg (SH), a pathogen that has shown great concern in the world poultry scene. The objective of this work was to evaluate the efficacy of *green yerba mate* (*Ilex paraguariensis* A.St.Hilarie, Aquifoliaceae) phytogenic additives in the control of SH UFPR1 strain in liver and cecum, as well as to evaluate the histological parameters applying the ISI methodology. The expression of immune cells: CD4+, CD8+, and peripheral sage macrophages were also evaluated. The free phytogenic additive showed a reduction of SH UFPR1 strain in cecum at 21 days, and statistically ($P < 0.05$) reduced the value of the liver ISI score when compared with the PC (positive control). The microencapsulation of this phytogenic additive reduces all this effect. In the cecum histology evaluation, NC (negative control) presented a lower ISI score at all times ($P <0.05$) compared to other groups as expected. Considering the results obtained in this research, the green yerba mate phytogenic additives have potential to be used in the control of *Salmonella enterica* serovar Heidelberg at the concentration of 0.1% applied in the feed.

Keywords: polyphenol-rich additive, immune system, antimicrobial, ISI methodology

1. INTRODUCTION

Salmonellosis is one of the major avian health problems and *Salmonella enterica* serovar Heidelberg (SH) has gained prominence in recent years due to its pathology and occurrence in Brazil (SANTIN et al., 2017; HAYASHI et al., 2018).

In an attempt to contribute to the control of the occurrence of *Salmonella* sp. in poultry production, several researchers have presented phytogenic additives with antimicrobial properties (RIZZO; MENTEN, 2010; BONA et al., 2012; CHENG et al., 2014; DIAZ-SANCHEZ; D'SOUZA, 2015; ABUDABOS et al., 2016). The mechanisms of action of the phytogenic additives are not completely elucidated, but the suggested mechanisms of action of plant extracts in the animal organism are changes in the intestinal microbiota, increase in the digestibility and absorption of nutrients, and antimicrobial and immunomodulatory effects (OETTING et al., 2006). The agronomic origin must be traced to maintain the standardization of actives (BOSSELMANN; GYLLING, 2013).

In an attempt to improve the stability of the phytogenic additives one can use the microencapsulation process, which consists of protecting the core material from the outside environment (DIAS et al., 2015) and many researchers have used maltodextrin as a coating material (AKHAVAN MAHDAVI et al., 2016; BALLESTEROS et al., 2017). Maltodextrins provide structural integrity to the final product and their incorporation reduces stickiness during drying (AUGUSTIN; HEMAR, 2009). The microencapsulation of additives, such as probiotics, has been employed to improve the stability of these products against the preparation process of the ration and conditions of the gastrointestinal tract (CORONA-HERNANDEZ et al., 2013). Still, according to these authors, the most common coating materials used are ionic polysaccharides, microbial exopolysaccharides and milk proteins, which present different physicochemical characteristics and may present mucous adhesion.

Many plant species have been studied as candidates for antimicrobial agents against *Salmonella sp.*, among them the yerba mate (*Ilex paraguariensis*

A.St-Hilarie, Aquifoliaceae) that showed a positive effect on *Salmonella* Enteritidis control *in vitro* (minimum inhibitory concentration 7.4 mg/mL) (GONZALEZ-GIL et al., 2014). The yerba mate aqueous extract was evaluated against the challenge of *Salmonella enterica* serovar Typhimurium and the metabolomic analysis of the mechanism of action concluded that yerba mate extract induced changes in central carbon metabolism in *Salmonella* Typhimurium, reduced catalase activity by means other than direct inhibition, and did not change membrane integrity despite a significant increase in the production of a cell wall precursor. Significant differences were observed in the global metabolic regulators alpha-ketoglutarate and acetyl phosphate, the energy-related molecule NAD+ and in an unexpected match to the antibacterial compound yohimbine (REMPE et al., 2017).

Many research on extracts and isolated compounds from yerba mate are been performed to benefit human health with a number of pharmacological applications, including: antioxidant, antimicrobial and anti-inflammatory (BURRIS, 2011).

The main of this research was the evaluated of green yerba mate phytogenic additives antimicrobial properties on *Salmonella enterica* serovar Heidelberg (SH) UFPR1 strain *in vitro* and *in vivo* in broilers, the histological I See Inside parameters at cecum and liver and expression of immune cells in peripheral blood of broilers.

2. MATERIALS AND METHODS

This trial was approved by the Institutional Animal Use Ethics Committee of Agricultural Sciences of the Federal University of Parana (Protocol 038/2016).

2.1 *In vitro* experiment

In order to evaluate and establish the antimicrobial activity and *in vivo* dose of the additive in this study, an *in vitro* analysis was carried out.

For this a mash broilers soybean and maize diet was sterilized by autoclave (121 ºC by 15 minutes) and after distributed in the following treatments: NC – negative control (without additive and SH non-inoculated); NC

+ PAM 0.1% (PAM - Phytogenic Additive Microencapsulated); NC + PAM 1.0%; PC – positive control (without additive and SH inoculated); PC + PAF 0.1% (PAF - Phytogenic Additive Free) and PC + PAF 1.0%. For the PC groups, 10 mL of 10^8 CFU/mL of SH and in NC groups 10 mL of sterilized water were spraying in 250 g of feed and be kept at 35-37º C for 24 h. After this period, the *Salmonella* isolation was according with described in 2.7.

2.2 *In vivo* experiment: Birds, Experimental Design, Diet and Housing

A total of 220 male broiler Cobb 500 from one to 21 d was used. The experiment followed a randomized factorial design 3x2, distributed in 4 cages of 9 birds, totalizing 36 replicate by treatment. Treatment description is present in table 1.

On the first day, four animals were euthanized and underwent necropsy before the experiment, and the liver and cecum were collected and evaluated for the presence or absence of *Salmonella* spp.

Prior to the beginning of the experiment, the experimental room was cleaned and disinfected, and *Salmonella* spp. the analysis was conducted to certify that the environment was free of this agent.

The birds were maintained in an isolated room at different cage by treatment at a comfortable temperature for their age, with feed and water *ad libitum*. During the first week (1-7d), the mean and standard error of the temperature was 30.12ºC ± 1.99; during the second week (8-14d), it was 28.10 ºC ± 1.19; and in the third week (15-21d), it was 27.02 ºC ± 1.00 within the values required by the COBB500 manual (2015). The diet was a corn and soybean-based mash that followed Brazilian nutritional recommendations for poultry (ROSTAGNO et al., 2011).

Table 1: Treatments description

Treatments	SH UFPR1 strain	Phytogenic additive added (kg/ton of feed)
NC	No	0
NC + PAM 0.1%	No	PA microencapsulated (1)
NC + PAF 0.1%	No	PA free (1)

PC	Yes	0
PC + PAM 0.1%	Yes	PA microencapsulated (1)
PC + PAF 0.1%	Yes	PA free (1)

2.3 Phytogenic additive

Phytogenic additive microencapsulated (PAM) and phytogenic additive free (PAF) patent number BR1020170096548 were products with a high level of total polyphenols, extracted from green yerba mate (*Ilex paraguariensis* A. St-Hil., Aquifoliaceae). The evaluation of the total polyphenol concentration in phytogenic additives was determined following the European Pharmacopoeia, 8th edition (European Pharmacopoeia, 2014). We used the Folin Ciocalteau (FC) reagent and a spectrophotometer reading SP-1105 (Spectrum, China) at 760 nm, using gallic acid as standard. This assay is based on the chemical reduction of the reagent containing sodium molybdate and sodium tungstate, which reacts with phenols and other substances such as ascorbic acid, aromatic amines, sugars and xanthines (SINGLETON et al., 1998). The doses used in *vivo* study followed the results of *in vitro* study. The value of total polyphenols for each dose evaluate *in vivo* were according to Table 2:

Table 2: Total polyphenols on phytogenic additives (GAE mg/g)

Phytogenic additive	**Total polyphenols (GAE mg/g)**	**Total polyphenols in 0.1% (GAE mg/g)**
Microencapsulated (PAM)	117.99	1.18
Free (PAF)	285.60	2.86

2.4 Challenge

At 3 days of age, the birds of treatments PC, PC + PAM 0.1% and PC + PAF 0.1% were orally inoculated with 10^8 CFU/mL of the SH UFPR1 strain. The SH UFPR1 strain used herein was isolated from commercial broiler carcasses in the South of Brazil (SANTIN et al., 2017) and the phenotypic and genotypic described the absence of several genes involved in antibiotics resistance as

amikacin, amoxicillin + clavulanate, ceftiofur, cephalexin, doxycycline and oxytetracycline and presents intermediary resistance to ampicillin + sulbactam, cephalothin, ciprofloxacin, enrofloxacin, and gentamycin and its resistance to short-chain organic acids (SCOA).

2.5 Evaluation of Live Production Performance

At the first day of age, birds were separated into treatments in a way that initial body weight average was similar in all cages selected for each treatment, in order to obtain equal initial body weight average per cage. Birds and feed were weighted weekly (zero, 7, 14 and 21 d) to evaluate feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR).

2.6 Sampling of the in vitro experiment

At 7, 14 and 21 days of birds age, 12 birds by treatment were euthanized by cervical dislocation and necropsied to sampling liver and cecum for bacteriological analysis, evaluation of histology by ISI methodology and peripheral blood was collected to evaluate the circulating immune cells (CD4+, CD8+, and macrophages) by flow cytometry.

2.7 Bacterial Isolation

Aseptically liver and the cecum were collected in a sterile plastic package for bacteriological analysis. The SH isolation was according to PICKLER et al., 2014. Briefly, the samples were weighed on the analytical balance and homogenized in 1:9 of 2% buffered peptone water (BPW). Further dilution was conducted by successively placing 1 mL of the solution in a test tube with 9 mL of 0.1% BPW until a 10^{-3} dilution was achieved. Then, 100 µL aliquots of each dilution were transferred to duplicate plates in Brilliant Green medium and uniformly spread with a sterile Drigalsky loop. The plates were incubated at 35°C for 24h, after which the typical colonies were counted. The initial 2% BPW was also incubated for 24h. If no typical *Salmonella* colonies had developed after the 24h incubation, 100 µL of the initial 2% BPW was placed in a tube with 10 mL of Rappaport-Vassiliadis broth and incubated at 42°C for 24h to confirm the negative and positive results of the samples.

2.8 Histopathology

Samples of liver and cecum were collected and fixed in Davidson´s solution (100 mL glacial acetic acid, 300 mL 90% ethyl alcohol, 200 mL 10% neutral buffered formalin and 300 mL distilled water) for at least 24 hours for the evaluation of histology. After fixation on Davidson´s solution, samples of liver and cecum were dehydrated, infiltrated and embedded in paraffin following common histological routine. Blocks were cut in 5 µm sections and stained with hematoxylin and eosin associated with Alcian Blue for goblet cells staining (RAPP and WURSTER, 1978). For cecum morphology, one slide and 30 intestinal villi per bird were observed in 10X magnification (using 20X and 40X magnification to confirm alterations) under an optical microscope (Nikon Eclipse E200, Sao Paulo, Brazil). For liver samples, 30 fields in 40X objective per bird were evaluated. The I See Inside (ISI) methodology used for liver histology evaluation was performed according to BELOTE et al., 2018 adapted from KRAIESKI et al., 2017. For cecum, it was established a histological evaluation of three parameters: (1) epithelial cells infiltration, (2) lamina propria inflammatory cell infiltration and (3) goblet cells proliferation according presented in Table 3.

Table 3: ISI histological alterations evaluated in cecum and liver.

Organ	Alterations	Impact factor (IF)		Score	Final score	Maximum[1] score
Cecum	Epithelial inflammatory cell infiltration	1	X	3	3	09
	Lamina propria inflammatory cell infiltration	1	X	3	3	
	Goblet cells proliferation	1	X	3	3	
Liver	Congestion	1	X	3	3	36
	Bile-duct proliferation	2	X	3	6	
	Immunne cells infiltration	1	X	3	3	
	Necrosis	3	X	3	9	
	Periocholangitis	3	X	3	9	
	Lymphocytic aggregate	2	X	3	6	

[1]Maximum score represents the sum of all alterations according to the formula ISI=Σ(IF*S), where IF = impact factor (previous fixed) and S = Score (observed) considering the maximum observed S. For example, the goblet cells proliferation has IF = 1; this number will be multiplied by the observed score (range from 1 to 3), if in a villus, a score S = 3 (maximum score) was observed for goblet cells proliferation, so the ISI for this parameter in the villi will be ISI = (1*3) = 3. The average of 30 villi in the cecum or 30 fields in the liver for each bird will be the final ISI value for each bird.

In this study, we adopted an impact factor (IF) value of 1 to all parameters available in the cecum because the digestion and absorption are not great as the small intestine and the functional implication of each alteration on it organs was not completely known.

2.9 Flow Cytometry

Blood was collected from 6 birds per treatment in tubes with heparin as an anticoagulant to evaluated the circulating immune cells (CD4+, CD8+, and macrophages) by flow cytometry. The flow cytometry was adapted from BEIRÃO et al., (2012), briefly, 50 µl whole blood were incubated for 30 minutes (37°C) with the specific antibodies and fixed with paraformaldehyde for 30 minutes at 4°C, then packed into cytometry tubes in the final volume of 2 ml of PBS. CD4+ helper T lymphocytes (LT) are responsible for orchestrating other immune response cells in pathogen eradication and are also very important in the activation of B lymphocytes (CIHAK et al., 1991), macrophages (QURESHI, 2003) or even CD8+ LT (LILLEHOJ, 1994).

The antibodies used in the present work are presented in table 4.

Table 4: Cells analyzed by flow cytometry

Cells	Antibodies
Peripheral Helper T lymphocytes ($CD4^+TCR\nu\beta1^-$)	CD4/TCRvβ1/CD45
Mucosa Helper T lymphocytes ($CD4^+TCR\nu\beta1^+$)	CD4/TCRvβ1/CD45
Mucosa Cytotoxic T lymphocytes ($CD4^-TCR\nu\beta1^+$)	CD4/TCRvβ1/CD45
Cytotoxic T lymphocytes ($CD8^+$)	CD8α/CD45
Monocytes (Kul^+)	Kul-1/CD45

2.10 Statistical Analysis

Data were presented as a mean ± standard error. At first, data normality was verified using the Shapiro-Wilk normality test. Rates were compared using one-way analysis of variance (ANOVA) followed by the Tukey test ($P < 0.05$) for parametric data. The CFU means obtained in microbiologic were log 10-transformed as well as the peripheral blood cell percentages evaluated by flow

cytometry. *Salmonella* negative and positive samples were compared using the chi-squared test.

Flow cytometry was analyzed by Fischer test (LSD) ($P < 0.05$). For performance, each cage was used as an experimental unit, while for the remaining analysis, each bird was used as an experimental unit.

All analyses were performed by Statistix 9 software for Windows.

3. RESULTS

3.1 *In vitro* analysis

The microbiologic results of *in vitro* study are present in Table 5. The negative control did not show *Salmonella* isolation and PC presented the highest CFU log result, as expected. The treatments PC + PAM 0.1% and PC + PAF 0.1% presented a statistically significant reduction in *Salmonella* CFU log (83 and 97% respectively) compare with positive control treatment (PC), however PC + PAM 1.0% and PC + PAF 1.0% showed lower SH reduction than treatment using 0.1% of phytogenic additive compared to PC (64 and 54% respectively).

Table 5: CFU/g Mean ± standard mean error of SH UFPR1 strain analysis in vitro.

Challenged	CFU/g (% of reduction compare to PC)
PC	35.333±6.6583 [a]
PC + PAM 0.1%	6.000±3.6056 [bc] (83%)
PC + PAM 1.0%	12.667±2.3094 [b] (64%)
PC + PAF 0.1%	1.000±1.7321 [c] (97%)
PC + PAF 1.0%	16.333±3.0551 [b] (54%)
P-value	0.0000

PC – positive control; PC + PAM 0.1% - phytogenic additive microencapsulated (PAM) 0.1%; PC + PAM 1.0% - phytogenic additive microencapsulated 1,0%; PC + PAF 0.1% - phytogenic additive free 0,1% and PC + PAF 1.0% - phytogenic additive free (PAF) 1,0%. [a.b.c.] Different letters in the same column indicate a significant difference ($P < 0.05$).

3.2 Evaluation of Live Production Performance

The body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) was not significantly different among treatments (data not shown). However, it was observed that the non-challenged group presented higher

BWG and worse FCR compared to challenge group into 1-14d (Figure 1 and Figure 2). Despite, we do not have many replicates to apply it as an animal zootechnical evaluation, these are useful as a clinical sign when compare challenged birds against non-challenged ones.

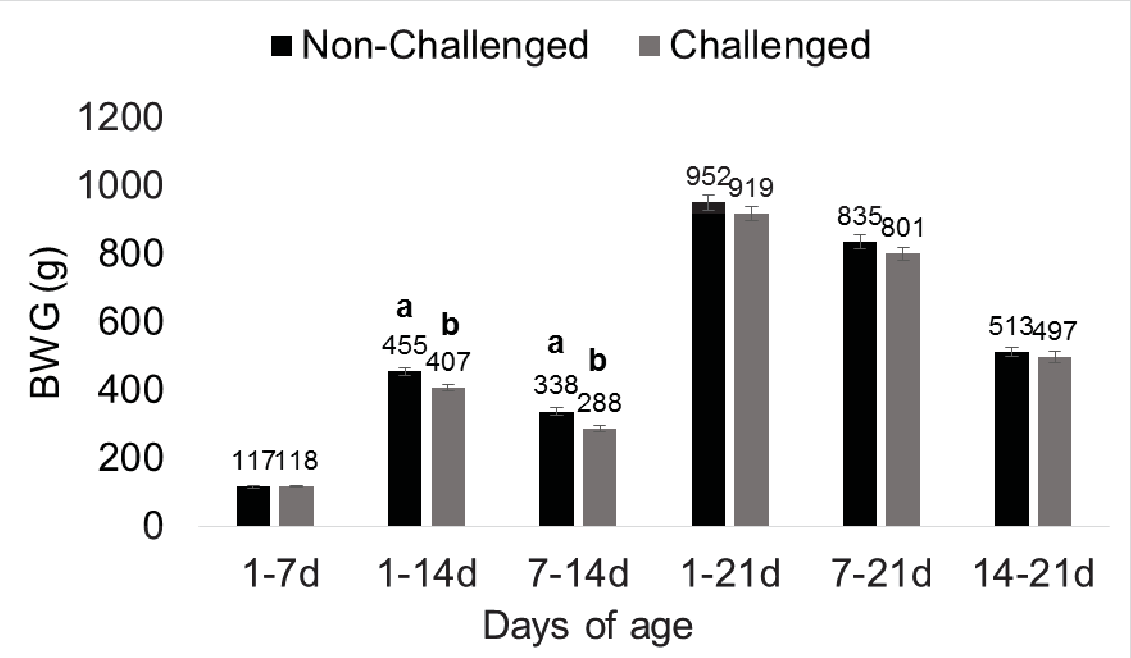

Figure 1: BODY WEIGHT GAIN (BWG, grams) non-challenged and challenged *Salmonella* Heidelberg SH UFPR1 strain. [a,b] Different superscript letters indicate significant difference ($P < 0.05$).

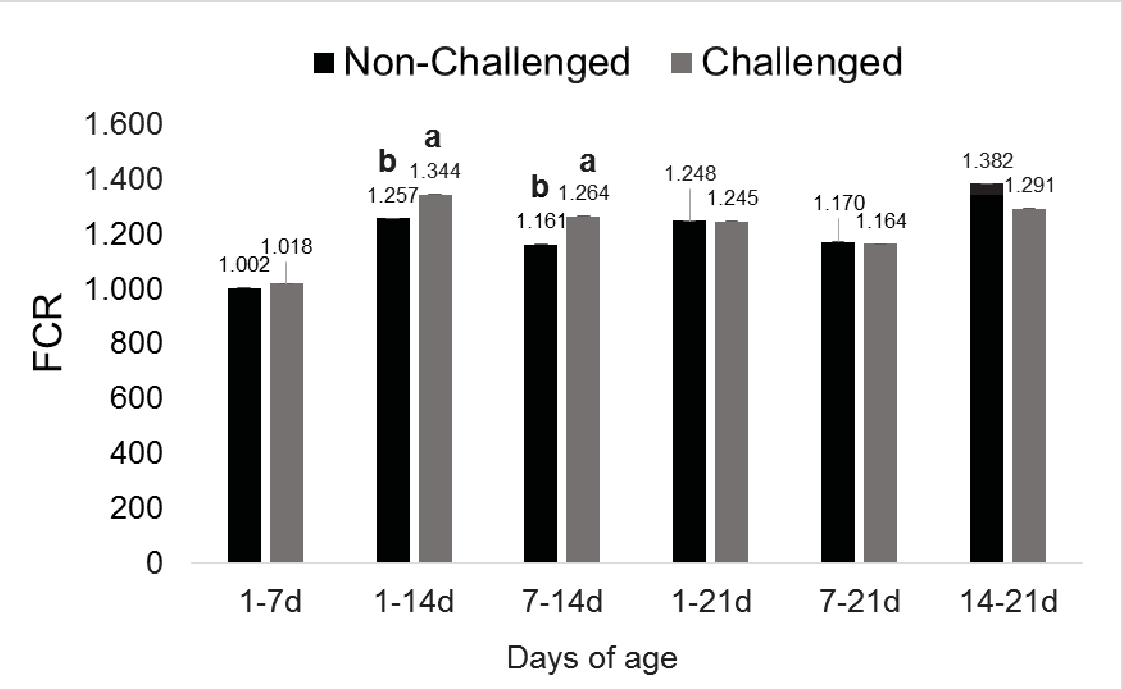

Figure 2: FEED CONVERSION RATIO (FCR) non-challenged and challenged *Salmonella* Heidelberg SH UFPR1 strain. [a,b] Different superscript letters indicate significant difference ($P < 0.05$).

3.3 *In vivo* microbiology results

At the evaluation of *Salmonella* isolation at *in vi*vo study, we observed, at 7 days that PAM group (PC + PAM 0.1%) showed higher cecum SH count than other groups (Figure 3). At 21 days, the PAF presented a significant reduction of the SH count on cecum comparing to PC (Figure 3). In liver, there are no typical *Salmonella* colonies at directly plate count and after the 24h incubation, there is no difference between treatments in terms of presence or absence of SH (no show data).

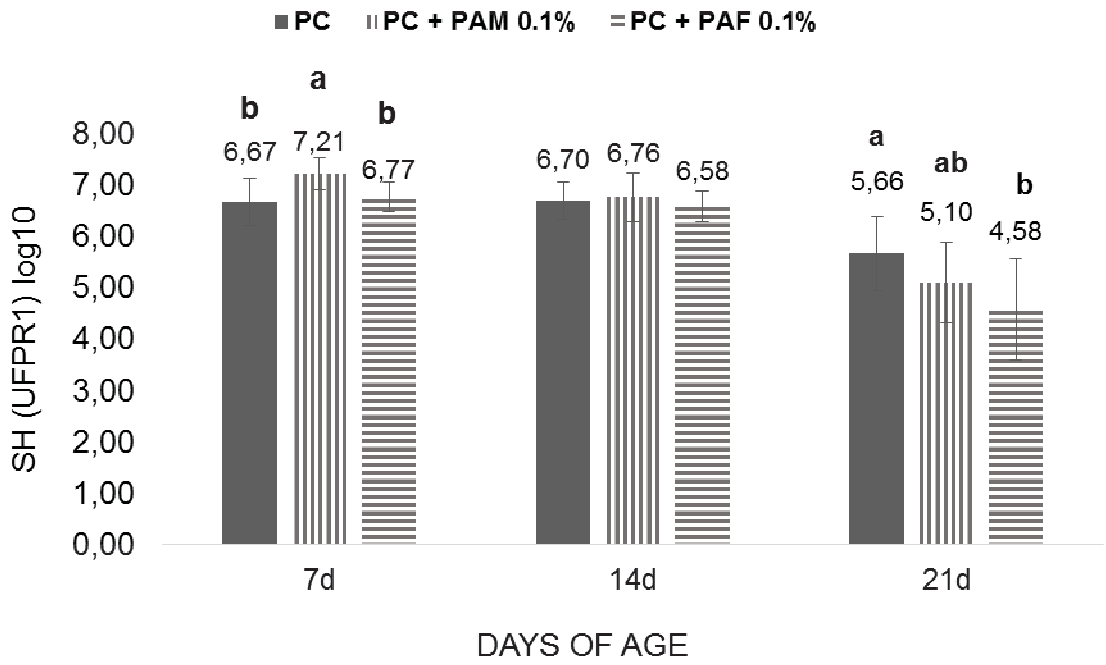

Figure 3: *Salmonella* Heidelberg SH UFPR1 strain log10 in cecum. PC – positive control SH UFPR1 strain; PC + PAM 0.1% - feed + 0.1% PAM + SH UFPR1 strain; PC + PAF 0.1% - feed + 0.1% PAF + SH UFPR1 strain. [a,b] Different superscript letters indicate significant difference ($P < 0.05$).

3.4 Histology

At liver histology, the non-challenged group presented lower ISI total score than the challenged group in all times (Figure 5) due to a higher score of congestion, immune cell infiltration, and periocholangitis. Comparing the ISI total score between non-challenged treatments at 7 days PAM and PAF treatments presented higher ISI score compared to NC treatments due to congestion and periocholangitis. In a challenged group, there is no difference between treatments. In the treatment, PAM challenged it was observed higher ISI score in the liver at 7 days compared to the same treatment non-challenged due to immune cell infiltration.

At 14 days, at the liver, ISI score the only significant difference observed is that PAF treatment showed lower ISI score compared to all challenged treatments due to a reduction in congestion. At 21 day, there is no difference between treatments at non-challenged groups but on challenged the PAM and PAF treatments presented lower liver ISI score compared to PC (Figure 6) due to a reduction on congestion and immune cell infiltration.

Considering cecum histology the challenged group presented higher ISI score compared to non-challenged due to an increase of lamina propria and epithelial inflammatory cell infiltration and goblet cells (Figure 9) in all periods.

At non-challenged groups, at 7 and 14 days, the PAM and PAF increase cecum score compared to NC due to increased inflammatory cell infiltration on lamina propria at 7 days and lamina propria and epithelial inflammatory cell infiltration at 14 days. At 21 days on non-challenged groups, only PAM increase ISI total score compared to NC due to an increase of lamina propria and epithelial inflammatory cell infiltration (Figure 10).

At challenged group the PAM treatment, increase the ISI total score in cecum due to increase in goblet cell and lamina propria and epithelial inflammatory cell infiltration in all periods compared to PC (Figure 10).

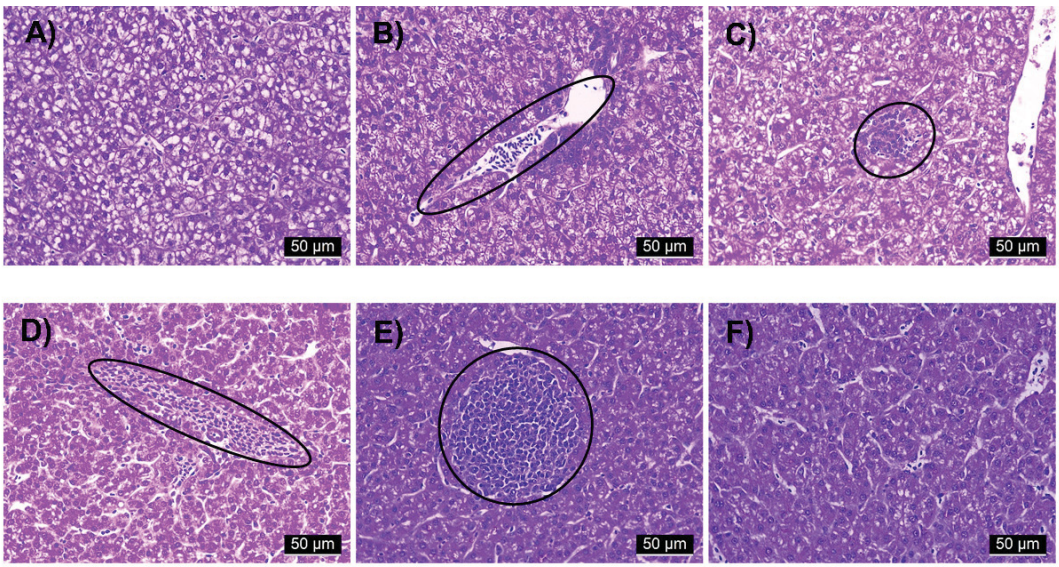

Figure 4: Photomicrographs of hematoxylin and eosin-stained chicken liver section. A) Normal liver in NC at 7d, 400X, B) Congestion (circle) in PAM group at 7d, 400X. C) Inflammatory cell infiltration (circle) in PAF group at 7d, 400X, D) Congestion (circle) in PC group at 21d, E) Inflammatory cell infiltration (circle) in PAM group at 21d and F) Normal liver in PAF group at 21d, 400X. These changes contributed to the highest ($P<0.05$) ISI score at 7d and 21d.

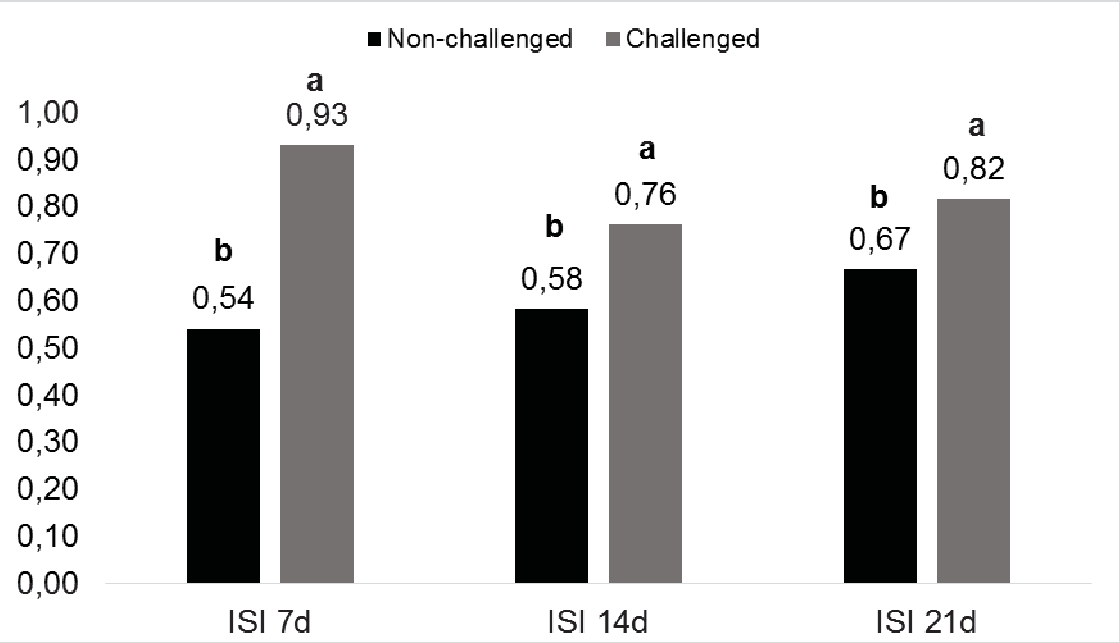

Figure 5: ISI total score in the liver, comparing non-challenge and challenge group. [a,b] Different superscript letters indicate significant difference (P < 0.05).

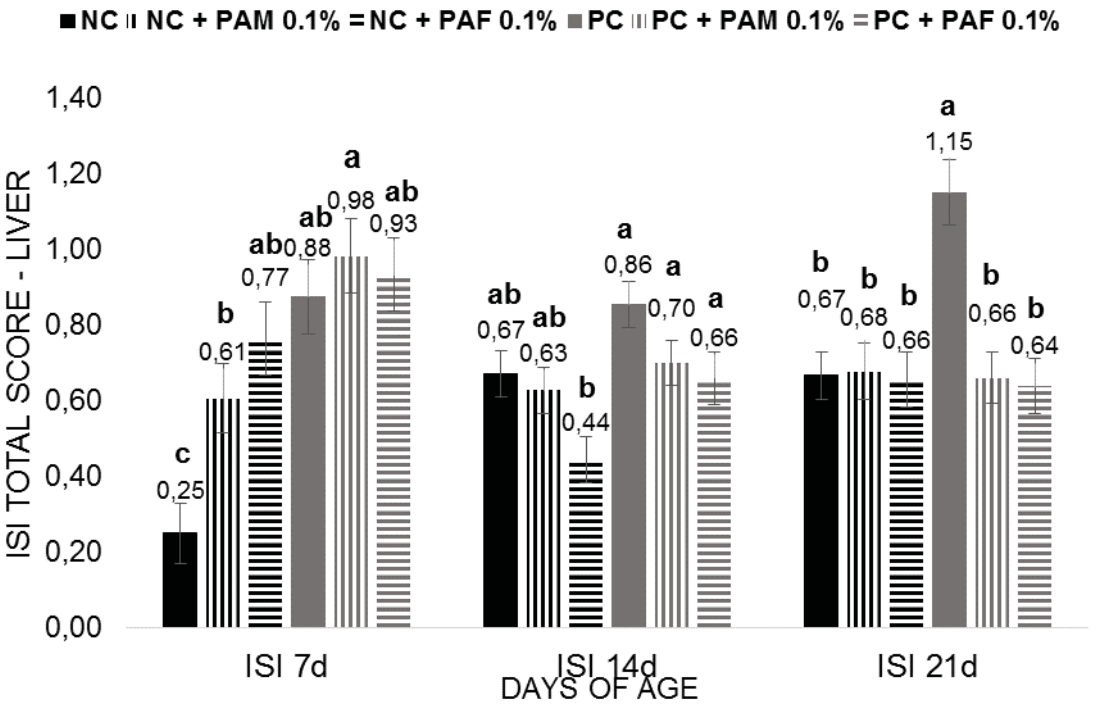

Figure 6: Liver histology using ISI methodology. NC – negative control; NC + PAM 0.1% – negative control + 0.1% PAM; NC + PAF 0.1% – negative control + 0.1% PAF; PC – positive control SH UFPR1 strain; PC + PAM 0.1% - feed + 0.1% PAM + SH UFPR1 strain; PC + PAF 0.1%- feed + 0.1% PAF + SH UFPR1 strain. [a,b,c] Different superscript letters indicate significant difference (P < 0.05).

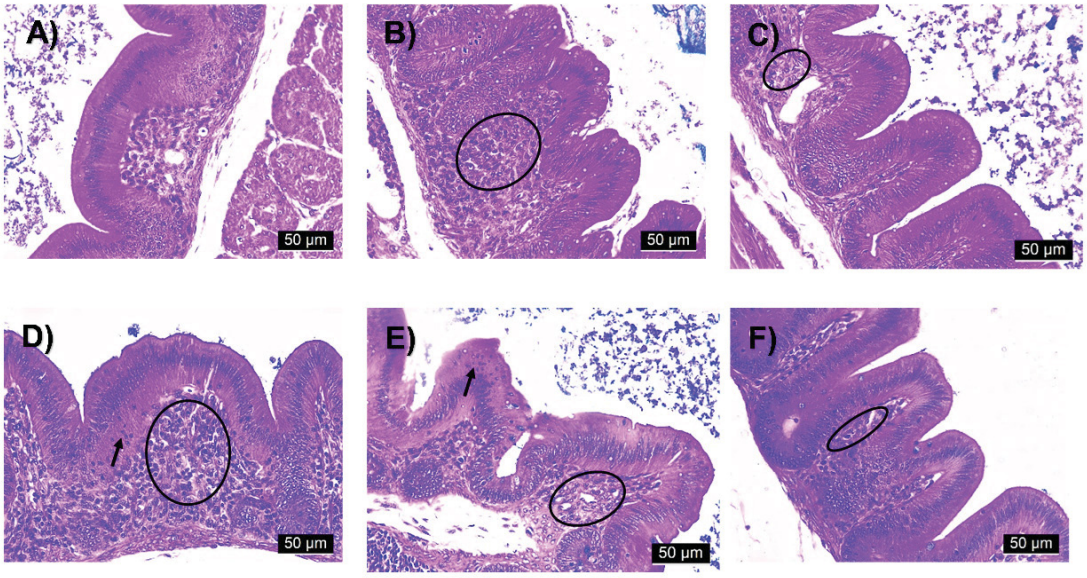

Figure 7: Photomicrographs of hematoxylin and eosin-stained broiler cecum section. A) Normal cecum of NC group at 7d, 400X. B) High ISI score of lamina propria inflammatory cell infiltration (circle) in PAM group at 7d, 400X. C) Low ISI score of lamina propria inflammatory cell infiltration (circle) in PAF group at 7d, 400X, D) High ISI score of lamina propria inflammatory cell infiltration (circle) and high ISI score epithelial inflammatory cell infiltration (arrow) in PC group at 21d, 400X, E) Low ISI score of lamina propria inflammatory cell infiltration (circle) and low ISI score epithelium inflammatory cell infiltration (arrow) in PAM group at 21d, 400X and F) Low ISI score of lamina propria inflammatory cell infiltration (circle) in PAF group at 21d. These changes were observed at 7d and 21d of age and contribute to the highest (P<0.05) ISI score.

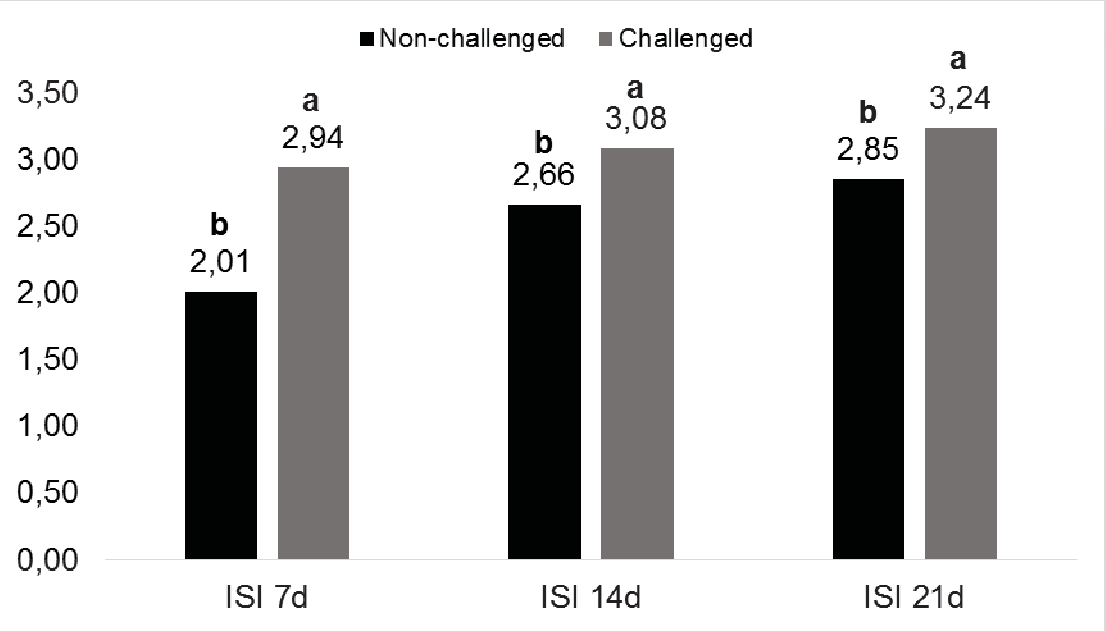

Figure 8: ISI total score in cecum, comparing non-challenge and challenge group. [a,b] Different superscript letters indicate significant difference (P < 0.05).

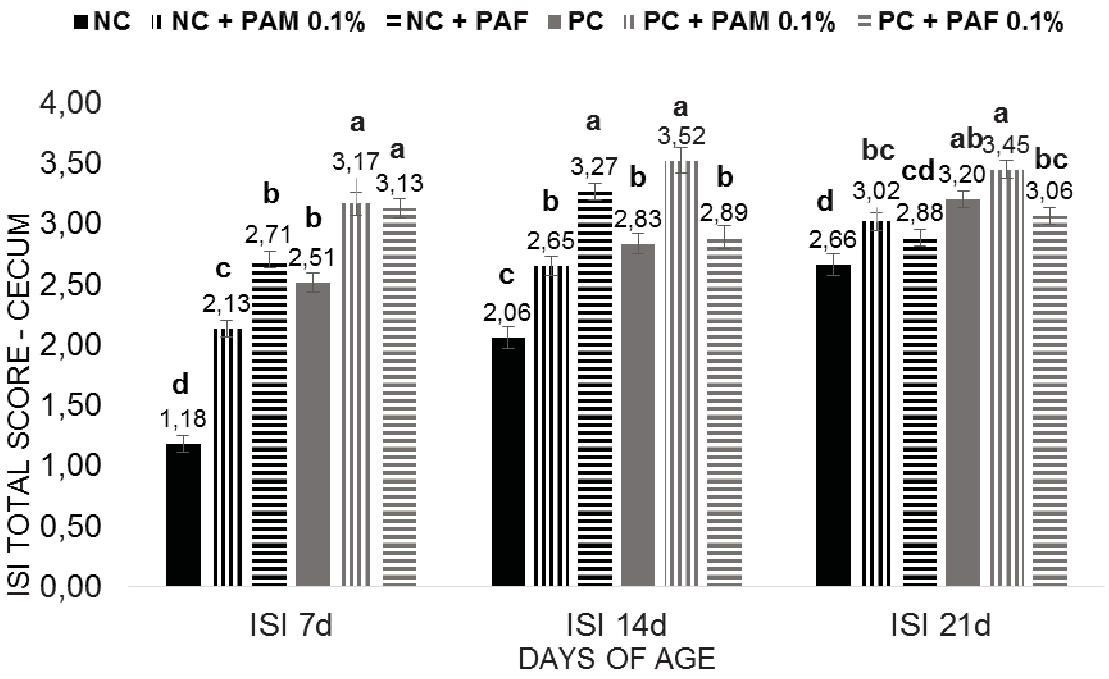

Figure 9: Cecum histology using ISI methodology. NC – negative control; NC + PAM 0.1% – negative control + 0.1% PAM; NC + PAF 0.1% – negative control + 0.1% PAF; PC – positive control SH UFPR1 strain; PC + PAM 0.1% - feed + 0.1% PAM + SH UFPR1 strain; PC + PAF 0.1%- feed + 0.1% PAF + SH UFPR1 strain. ^{a,b,c,d} Different superscript letters indicate significant difference ($P < 0.05$).

3.5 Flow cytometry

Results of flow cytometry analysis in peripheral blood demonstrated that at 14 days CD4+TCRvβ1^{-} and CD4^{+}TCRvβ1^{+} results were higher at PAF and NC compared to PC and PAM (Table 6). The CD4^{-}TCRvβ1^{+} at PC was lower than PAM. At 21 days, PAF presented lower CD8+ than PC group and there is no difference for NC and PAM. For KUL+ the group PAM presented higher KUL+ results than NC (Table 7).

Table 6: Flow cytometry (CD4^{+}TCRvβ1^{-}, CD4^{+}TCRvβ1^{+} and CD4^{-}TCRvβ1^{+}) at 14 days. NC – negative control; PC – positive control SH UFPR1 strain; PAM – feed + PAM 0.1% + SH UFPR1 strain; PAF - feed + PAF 0.1% + SH UFPR1 strain.

Blood	**14 days**		
Treatments	**CD4^{+}TCRvβ1^{-}**	**CD4^{+}TCRvβ1^{+}**	**CD4^{-}TCRvβ1^{+}**
NC	3.30±0.33 ^{ab}	13.63±1.15 ^{a}	3.54±0.38 ^{ab}
PC	2.47±0.34 ^{bc}	9.01±1.00 ^{bc}	3.17±0.29 ^{b}
PAM	2.13±0.25 ^{c}	7.96±0.53 ^{c}	4.33±0.55 ^{a}

PAF	3.61±0.56 [a]	11.79±1.14 [ab]	3.62±0.29 [ab]
P-value	**0.0396**	**0.0013**	**0.1000**

Table 7: Flow cytometry (CD8+ and KUL+). NC – negative control; PC – positive control SH UFPR1 strain; PAM – feed + PAM 0.1% + SH UFPR1 strain; PAF - feed + PAF 0.1% + SH UFPR1 strain.

Blood	**21 days**	
Treatments	**CD8+**	**KUL+**
NC	7.46±1.20 [b]	8.28±0.80 [b]
PC	10.27±0.47 [a]	11.03±1.09 [ab]
PAM	8.17±0.58 [ab]	11.78±1.03 [a]
PAF	6.29±0.85 [b]	10.34±0.83 [ab]
P-value	**0.0229**	**0.1000**

[a,b] Different superscript letters indicate significant difference at $P < 0.05$. Fischer test.

4. DISCUSSION

The microbiologic results of *in vitro* study showed that PAM or PAF 0.1% presented a significant ($P < 0.05$) reduction in *Salmonella* CFU log compare with positive control treatment (PC), but at 1.0% the reduction was lower than 0.1%. With these results, we establish 0.1% (100 mg/100g) as the level to be used in the *in vivo* experiment.

The *in vitro* result could suggest a quorum sensing effect. Gram-negative bacteria use quorum sensing communication circuits to regulate a diverse array of physiological activities (MILLER, 2001) and the 1.0% of product in medium of both phytogenic additives (PAM and PAF) changes medium culture conditions and could favoring the release of chemical signal molecules called autoinducers which may have contributed to the increase in the population density of *Salmonella* Heidelberg.

In addition, a study of *Salmonella enterica* serovar Typhimurium (ST) demonstrated that the transcriptome of *Salmonella* is medium-dependent and that the type of bacterial culture medium can promote profound differences in the global transcriptome after *in vitro* growth (BLAIR et al., 2013). With the same ST, authors suggest that the specific media growth conditions impact how the bacteria interact with host cells (SRIDHAR; STEELE-MORTIMER, 2016).

Some studies of analyses of dose-response curves to compare the antimicrobial activity verified that some compound, like peptide KLK-L5, change the idealized dose-response curves, and increased the % bacterial growth of *E.coli* from the concentration of 1.0 µM compared to 1.5 µM (RAUTENBACH et al., 2006).

At *in vivo* study, the productive parameters are evaluated only as clinical sign once there are few replicate numbers for zootechnical performance evaluation. The use of the PAM and PAF additives did not change the live production performance parameters, including body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) in relation to the negative control group (data not show). It was observed the influence of challenge on performance into 1-14d where BWG was higher and FCR was lower in the non-challenge group compared to the challenge group. This result is different from studies of Muniz et al. (2015), Santin et al. (2017), Hayashi et al., (2017) in which *Salmonella* challenge did not influence the performance of broilers, however each one of the past studies used different challenge level (10^7 CFU instead of 10^8 in the present study) or different periods of challenge (15 days, 1 day and 3 days, respectively). The effect on performance on the present study was only observed at 14 days, a period that was not performed at the past cited studies. According to the challenged at present study (3 days), this could be related to inflammatory processes observed in ISI score on cecum of challenged birds compared to non-challenged one and could be a result of the cost of immune response (KLASING, 2004). The interaction involving nutrition, challenge, and immunity is a strategic determinant in animal health (KOGUT; KLASING, 2009).

At the evaluation of *Salmonella* isolation at *in vivo* study showed that PAM 0.1% group increase significant the SH count compared with other groups at 7 days. It could be explained by the effect of maltodextrin on cecum SH colonization, as reported in a previous study in mice (NICKERSON et al., 2014) which showed also increased cecum bacterial loads after a short time ingestion of maltodextrin but did not increase the systemic disease. These results suggested that the consumption of maltodextrin alters intestinal homeostasis in favor of *Salmonella* colonization at this age. In the same time, PAM increase cecum ISI score compared to NC due to increasing inflammatory cell infiltration

on lamina propria but did not present significant difference on blood immune cells by flow cytometry (CD4+, CD8+, and KUL+) at 7 days. However, at 14d PC and PAM groups presented lower results of CD4+TCRvβ- and CD4+TCRvβ+ than NC indicating that the cells can migrate to the site of action and decreased in the circulation. Pickler et al. (2014), affirmed that in *Salmonella* Enteritidis infections in chickens resulted in the reduction of immune cells in the blood flow due to the migration of these cells to the site of action. Considering CD4-TCRvβ+ result at 14 days, the PAM group presented significantly higher value than PC group, suggesting that the PAM can be promoted higher challenge than PC.

At 21 days, PAF presented a significant reduction of the SH count comparing to PC. This repeat the same antimicrobial action observed *in vitro*. At the same time, PAF present significant lower ISI score in liver and cecum and a lower count of blood CD8+ comparing to PC. This result suggests an antimicrobial and anti-inflammatory effect of the free phytogenic additive (PAF). The effect on *Salmonella* control could be attributed to the levels of total polyphenols present in the PAF composition. A study evaluating the use of blueberry (*Vaccinium corymbosum* L.) extracts against the growth of *Salmonella* Enteritidis showed that phenolic compounds in the extracts such as chlorogenic acid, quercetin, ellagic acid, and quercetin-3-galactoside were the active antimicrobial compounds in the blueberry extracts (SHEN et al., 2014). The results of the present work demonstrated that the phytogenic additive based on green yerba mate showed antimicrobial efficacy against S. Heidelberg, contrary to the lack of activity of the yerba mate extract present in other *in vivo* assay against S. Enteritidis (GONZALEZ-GIL et al., 2014). This difference could be explained by the difference in *Salmonella* serovar between the studies, as well as, in actives concentration of the evaluated extracts. When a phytogenic additive is introduced into the feed, it is expected a change in the microbiota of the host, and however we did not evaluate it in the present study, this also should be evaluated in future to clarify the *Salmonella* control effect in PAF group.

In all periods, liver and cecum ISI score for the non-challenged group was significantly lower than the challenged group and these results agree with Santin et al., (2017) in which SH UFPR1 strain infection produced mild

histologic alterations in liver and cecum compared to non-challenged birds, mainly associated with inflammatory processes. At liver histology, the congestion, immune cells infiltration, and periocholangitis was the cause of higher ISI score in the challenged group compared to non-challenged. At the cecum, goblet cells, epithelial and lamina propria inflammatory cell infiltration it was also observed in challenged compared to the non-challenged group at all times. Presence of congestion and inflammatory cell infiltration suggested defense mechanisms. According Bromm; Kogut (2018), inflammatory process occur naturally and are essential to immune response, besides this, increased pro-inflammatory mediators have been associated with enhanced resistance to a range of important poultry pathogens.

It was possible observe that the use of the phytogenic additives (PAM and PAF) increased the liver and cecum ISI total score comparing to NC at 7 days and 14 days and this can be caused by the presence of tannins, which are considered as anti-nutritional factors in broilers by different authors (HOUSHMAND et al., 2015 and MORAES et al., 2018). According to Keshavarzi et al. (2017), dietary tannins rich caused adverse effects like had higher liver weights at 21 d of age, but they concluded that the tannins effects reduced with age, the same observed in this study. Dietary components can regulate the physiological functions of the body; interacting with the immune response is one of the most important functions of nutrients (KOGUT; KLASING, 2009).

5. CONCLUSIONS

The *in vitro* microbiological evaluation of phytogenic microencapsulated (PAM) and free (PAF) additives demonstrated efficacy at 0.1% concentration against the challenge of *Salmonella* Heidelberg SH UFPR1 strain.

In the *in vivo* experiment, the use of the PAM and PAF additives did not change the live production performance parameters, including body weight gain (BWG), consumption and feed conversion ratio (FCR) in relation to the negative control group (data not show). It was observed that challenge reduced BWG and increase FCR in broilers.

With the accomplishment of this study it was possible to conclude that phytogenic additive microencapsulated (PAM) did not present the expected

results, possibly because it contains maltodextrin and lower content of total polyphenols comparing to free phytogenic additive (PAF). The phytogenic additive free (PAF) presented a potential use as antimicrobial against SH UFPR1 strain, because at 21 days, PAF presented a significant reduction of the SH count, presented a significant lower ISI score in liver and lower result at blood CD8+ comparing to PC. This result suggests an antimicrobial and anti-inflammatory effect of the free phytogenic additive (PAF) in broilers.

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FINAL CONSIDERATIONS

The present research work contributed to the advancement of the national research in the field of phytogenic additives, which has shown promise in the attempt to control pathogens such as *Salmonella enterica* serovar Heidelberg. In its first chapter, it was possible to establish, through an experimental design and application of surface response methodology, the optimal conditions for the extraction of total polyphenols of green mate, with the following results: temperature of 50.00 °C, enzyme concentration of 168 FGB/100 g, reaction time of 120 minutes and pH 4.50. In the second chapter, microparticles of a phytogenic additive were evaluated, with or without barium sulfate, by radiological evaluation. It was observed that the microencapsulated additive presented resistance to passage through the crop and releasing the contents at the beginning of the jejunum. The third chapter describes the *in vitro* and *in vivo* studies conducted with the objective of evaluating the activity of phytogenic additives in the control of *Salmonella enterica* serovar Heidelberg (SH UFPR1 strain) in the liver and cecum. The phytogenic additive microencapsulated (PAM) did not present the expected results, possibly because it contains maltodextrin and lower content of total polyphenols comparing to free phytogenic additive (PAF). The phytogenic additive free (PAF) presented a potential use as antimicrobial against SH UFPR1 strain, because at 21 days, PAF presented a significant reduction of the SH count, presented a significant lower ISI score in liver and lower result at blood CD8+ comparing to PC. This result suggests an antimicrobial and anti-inflammatory effect of the free phytogenic additive (PAF) in broilers.